UNIVERSITY OF LIVERPOOL

TOWARDS THE PREDICTION AND DIAGNOSIS OF DRUG HYPERSENSITIVITY REACTIONS

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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DECLARATION

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree.

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ABBREVIATIONS

ABC abacavir

ADR adverse drug reaction

APC antigen presenting cell

ATP adenosine triphosphate

AX amoxicillin

BB bandrowski's base

BP benzylpenicillin

BSA bovine serum albumin

CBZ carbamazepine

CCR(#) chemokine receptor (#)

CD(#) cluster of differentiation (#)

CLA cutaneous lymphocyte antigen

CPM counts per minute

CRM chemically reactive metabolite

CTL cytotoxic T lymphocyte

CTLA-4 cytotoxic T-lymphocyte-associated protein-4

CYP cytochrome p450

DAMP damage associated molecular pattern

DC dendritic cell

moDC mature dendritic cell

DDS dapsone

DDS-NO dapsone-nitroso

DHR drug hypersensitivity reaction

DHS drug hypersensitivity syndrome

DILI drug induced liver injury

iDILI idiosyncratic drug induced liver injury

DMSO dimethyl sulfoxide

DNA deoxyribose nucleic acid

DRESS drug reaction with eosinophilia and systemic symptoms

EBVs epstein-barr virus derived B-cells

EDTA ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

ELISpot enzyme-linked immunosorbent spot assay

FACS flurorescence assisted cell sorting

FasL fas ligand

FBS foetal bovine serum

FCS foetal calf serum

FDA federal drug administration

FITC fluorescein isothiocyanate

FoxP3 forkhead box P3

FSC forward scatter

GB granzyme B

M-CSF macrophage colony-stimulating factor

GM-CSF granulocyte-macrophage colony-stimulating factor

GWAS genome-wide association study

HBSS hank's balanced salt solution

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

His histidine

HIV human immunodefficiency virus

HLA human leukocyte antigen

HPLC high performance liquid chromatography

HSA human serum albumin

IDR idiosyncratic drug reactions

IFN-γ interferon gamma Ig immunoglobulin

IL(#) interleukin (#)

JAK janus activated kinase

LC liquid chromatography

LCK lymphocyte-specific protein tyrosine kinase

LPS lippopolysaccharaide

LTT lymphocyte transformation test

MACS magnet assisted cell sorting

MAPK mitogen activated protein kinase

MFI mean fluorescence intensity

MHC major histocompatability complex

MS mass spectrometry

NF-kb nuclear factor kappa beta

NHS national health service

NK natural killer

NKT natural killer T cell

NRF2 nuclear factor (erythroid-derived 2)-like 2 OR odds ratio

PAMP pathogen associated molecular pattern

PBMC peripheral blood mononuclear cells

PBS phosphate buffered saline

PD-1 programmed death protein 1

PD-L1 programmed death protein ligand 1

PFA paraformaldehyde

PHA phytohaemagglutinin

Pip piperacillin

ROS reactive oxygen species

RPM revolutions per minute

SCAR severe cutaneous adverse reactions

SDS-PAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis

SFU spot forming units

SI stimulation index

SJS stevens-johnson syndrome

SMX sulfamethoxazole

SMX-NHOH sulfamethoxazole hydroxylamine

SMX-NO sulfamethoxazole nitroso

SSC side scatter

STAT signal transducer and activator of transcription

T-bet T box transcription factor

TCR T cell receptor

TEMRA T-effector memory cells re-expressing CD45RA

TEN toxic epidermal necrolysis

Th T-helper

TLR toll-like receptor

TNF tumour necrosis factor

Treg T regulatory cell

TT tetanus toxoid

WHO world health organisation

PUBLICATIONS & CONFERENCES

Published Papers

(* = co-author)

Implications of HLA-allele associations for the study of type IV drug hypersensitivity reactions.

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Up-Regulation of T cell Activation MicroRNAs in Drug-Specific CD4(+) T cells from Hypersensitive Patients.

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Development of a screening assay system to predict the intrinsic immunogenicity of a drug or compound.

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J. Watkinson, L. Faulkner, A. Gibson, B Kevin. Park, DJ. Naisbitt.

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ABSTRACT

Adverse drug reactions (ADRs) are becoming an increasing burden for both the healthcare system and the pharmaceutical industry. Indeed, up to 6.5% of all hospital admissions are attributed to ADRs. Drug hypersensitivity reactions (DHRs) are of particular interest to us – ADRs with an immune aetiology - especially T cell mediated delayed type reactions. They are idiosyncratic in nature, and as such are not predictable based on the pharmacology of the drug. Genetic studies have elucidated associations between specific HLA alleles and DHR, but in most cases a reaction is not observed in the patient. *In vivo* drug challenge tests have been used to diagnose and predict the likelihood of an DHR, but are not always appropriate due to their invasive nature and inaccurate predictive capabilities. What is desperately needed in order to advance the field and prevent DHR are accurate, repeatable, and reliable *in vitro* tests for both the diagnosis and prediction of these reactions.

Whilst dendritic cell T cell co-culture systems enable us to determine the ability of a compound to prime and activate naïve T cells, there are some problems that must be addressed. Existing assays do not take into account the precursor frequency of naïve T cells for a specific drug. Thus, naïve T cell priming to certain drugs cannot be detected, as there not enough T cells to reach the precursor frequency required for activation. The assay is also too complicated, with cells being cultured and moved between plates multiple times for each assay. Additionally, only one donor at a time can be analysed. For this reason, within this thesis I have generated two new assays; the T cell multi-well assay (T-MWA), and the T cell multi-donor assay (T-MDA). Post-dendritic cell generation, the T-MDA and T-MWA take place in a single 96-well plate, miniaturising and streamlining the assay. The T-MDA allows for the comparison of up to 16 donors in a single assay, with positive responses being able to be examined in further detail by the T-MWA. In each assay nitroso sulfamethoxazole (SMX-NO) led to positive priming in all donors (either SI>1.5 or $p<0.05$ in Mann-Whitney statistical test), whilst the β-lactam antibiotic piperacillin led to positive priming in only one donor in each assay. Bandrowski's base (BB), an oxidation product of the contact allergen *p*-phenylenediamine led to positive priming in 4 out of 8 donors. The T-MWA allows for the analysis of 40 wells per drug treatment (opposed to 3), which accounts for the T cell precursor frequency associated to a drug reaction. The T-MWA was used to establish naïve T cell priming against SMX-NO, BB, piperacillin, and sulfamethoxazole (SMX). The greatest level of priming in terms of both statistical significance and the strength of response was observed to SMX-NO and BB as these regularly had good or strong responses (SI>2) and dot plots of all wells had $p<0.05$. Priming was still achieved to piperacillin and SMX, albeit with fewer responsive wells responding, with weaker levels of proliferation. This enables SMX-NO to be used as a positive control, and something by which in the future researchers can compare the immunogenicity of other drugs.

Despite the success of the T-MDA and T-MWA, it was important to understand whether the assay recreates accurate immune regulation, and whether this would change dependent on the test drug and have a bearing on the observed results. T regulatory cells (Tregs) as well as programmed death-1 (PD-1) and cytotoxic T lymphocyte-associated protein-4 (CTLA-4) all have immunosuppressive actions on the immune system and can determine whether or not T cell activation occurs. Tregs

were generated during the course of priming in 3 out of 3 donors to both SMX-NO (Tregs <1% of CD4+ goes to mean of 3% of CD4+ cells) and piperacillin (Tregs <1% of CD4+ goes to mean of 2.5% of CD4+ cells); whilst PD-1 and CTLA-4 expression overall increased in all donors. Upon blockade of PD-1 and CTLA-4 in the T-MWA, the strength of the response increased, but negative priming was not reversed upon blockade of the co-inhibitory pathways due to increased background proliferation ($P>0.05$).

The lymphocyte transformation test (LTT) is the current gold standard of *in vitro* diagnostic assays, but its applicability domain is limited in vulnerable paediatric patient cohorts who often suffer DHR due to their extended exposure to multiple drugs. Out of 13 patient samples tested for drug specific PBMC proliferative responses: 8 of them resulted in negative results ($p>0.05$), despite the patient presenting with suspected DHR from clinical observation. Four resulted in a single potential culprit drug being positive in LTT, whilst 1 flagged multiple potential culprit drugs. This makes determining the correct drug to withdraw extremely difficult in cases of multiple drug treatment. Additionally, the LTT takes 6 days to complete, which is too long for a diagnostic assay. miRNA are known to regulate a range of biological processes and regulate expression of up to 50% of genes. They are also known to play a regulatory role in the immune system and may determine whether or not activation occurs. miR 9, 18, 21, and 155 are known to be upregulated upon immune activation. We used a miR upregulation assay whereby upregulation of these known miR could be quantified and correlated with T cell activation in 3 days instead of 6. miR 9, 18, 21, and 155 were differentially upregulated in antigen specific T cell clones and patient PBMC all of which were positive by LTT or ELISpot, proving the concept that miR upregulation could be used in the future to determine T cell activation more rapidly than the LTT.

These studies were successful in bringing about improvements to *in vitro* assays such as the naïve T cell priming assay and LTT. However, further studies into the immune regulatory pathways involved at the cellular and transcriptional level will be the key to improving and therefore moving towards truly dependable predictive and diagnostic *in vitro* assays for DHR.

1 GENERAL INTRODUCTION

Due to rapid increase in novel drugs being developed for a plethora of illnesses, the issue of adverse drug reactions (ADR) to these drugs are becoming more and more common [1]. These reactions are a bigger problem than most realise; with some reactions resulting in high morbidity illnesses, and in extreme cases, mortalities. In fact, adverse drug reactions have been associated with 6.5% of all hospital admissions, and over 100,000 admissions to hospital are due to drug exposure [2]. This problem is increasing in severity for both the patients, and the hospitals that deal with an increasing number of patients taking up hospital beds due to ADR. Indeed, it was estimated that the increase in additional hospital bed days directly due to ADRs could be placed up wards of 2000 at any single point in time. More shockingly, the equivalent of ten, eight hundred bed hospitals could be filled when combining the above data with total bed days for in-patients due to an ADR [3].

Drug hypersensitivity reactions (DHR) are ADRs with an immunological aetiology. It is abundantly clear that it is necessary to find ways to determine why these ADRs, in order that we can prevent them where possible, and effectively treat them in a time efficient manner as soon as possible in other instances. Some of the current methods of diagnosis of drug hypersensitivity reactions (DHR) such as intradermal, skin prick and patch tests [4] are both inconvenient for the patient due to their invasive nature, and not altogether useful due to the potential for false positives stemming from low sensitivity of the tests, or cross-reactivity of certain antibiotics, which can again lead to false results. It is evident that better testing for ADR and DHR must be established, and that a less invasive method would be better for both patients and clinicians.

The use of *in vitro* testing is something that is increasing in popularity for its non-invasive nature which can be carried out in a more convenient and less time-consuming environment for the patient. The development of diagnostic tests such as

the lymphocyte transformation test (LTT) has gained popularity, particularly in research; but the development of a working, predictive *in vitro* assay remains the end-goal. It would be remiss to discuss the various forms of *in vitro* assays for diagnosis and prediction of DHR, without further outlining what adverse drug reactions and drug hypersensitivity reactions are, and how they cause the need for the development of these assays.

1.1 ADVERSE DRUG REACTIONS

1.1.1 DEFINITION

The definition of an ADR was first established in 1972 by the World Health Organisation, which stated that an ADR could be defined as 'a response to a drug which is both noxious and unintended, and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function' (WHO, 1972). This classification was well-accepted and understood for almost 30 years. However, a new definition was put forward in 2000 by Edwards and Aronson which stated, 'an appreciably harmful or unpleasant reaction, resulting from an intervention related to the use of medicinal product, which predicts hazard from the future administration and warrants prevention or specific treatment, or alteration of the dosage regimen, or withdrawal of the product' [5]. This updated definition of ADRs has since become the more accepted phrasing, since it takes into account any wording inaccuracies from the original definition; such as the inclusion of the word 'noxious'. It was determined that the use of the word noxious may mislead people into the belief that all ADRs are severely harmful, in spite of the fact that some ADRs remain merely inconvenient, but not deeply harmful.

1.1.2 CLASSIFICATION

The classification of ADRs - much like the definition of them - was a progression from an original idea, which was later expanded upon by the discontent of Edwards and Aronson. Type A reactions are the most commonly observed drug reaction, due to their nature as an on-target reaction which is often dose-dependent. This type of reaction is predictable from the known pharmacology of the drug. Type A reactions occur usually as a consequence of drug overdose, drug-drug interactions if the patient is prescribed more than one drug, or as a result of compromised metabolism. Type B

reactions on the other hand, are often termed as idiosyncratic, dose-independent and bizarre due to the inability to predict them from the known pharmacology of a drug. Type B reactions are considered more dangerous as they are not predictable and often do not stop upon cessation of treatment, as they routinely activate the patients' immune system which links them in with DHR. It could be argued that much like the definition of an ADR, the classification of them have become outdated also.

With the relatively recent discoveries of HLA-linked drug reactions such as HLA-B 57:01 in abacavir [6–8], any new classification system must take into account these important discoveries. In 2000, Edwards and Aronson added four more classifications to the original idea of Type A and B reactions. These were dose and time related Type C (chronic), time related Type D (delayed), Type E end of use of the drug, and type F which is related to therapeutic failure of the drug [5]. Aronson added a further classification of ADRs known as DoTS, which related to the dose, timing and susceptibility factors, which tied in with reactions that were idiosyncratic in nature but were seen as dose-dependent [9].

1.1.3 IMPORTANCE

It is clear that the elucidation of the mechanisms of ADRs must be a key goal in current scientific research, in order that they become preventable, or at least more quickly and efficiently treatable. As previously stated, ADRs cause great damage each year via a plethora of avenues. They first and foremost, cause great problems for the patient on the drugs; whether the symptoms are either inconvenient or life-threatening. ADRs have been implemented in over 100,000 deaths per annum, solely in the United States [10] and elsewhere been classified as one of the most common factors associated to the death of patients [11]. Once again, these statistics clearly outline the potential danger of ADRs, and the severe effect they may have on countless individuals. Mortality due to

ADRs are not the sole concern; as ADRs often result in high morbidity illnesses that leave patients needing hospital stays, or at the very least, visits. The financial strain on the National Health Service (NHS) attributed to ADRs remains an important concern as up to 10% of impatient costs can be linked to drug reactions [3].

1.2 DRUG HYPERSENSITIVITY REACTIONS

DHR can also be described as drug allergy and are linked to Type B ADRs. Like Type B ADRs, DHRs are unpredictable and idiosyncratic in nature; with reactions occurring at normal doses of the prescribed drug. The defining factor that distinguishes a DHR from an ADR is the triggering of the host's immune response. Therefore, a DHR can be defined more concisely as an adverse drug reaction that has an immune based aetiology to normal doses of safe therapeutic agents.

One of the main causes of cases of DHR lies in response to the beta lactam classes of antibiotics [12], with drug-drug interactions playing a role in additional incidences of hypersensitivity. Indeed, patients who are exposed regularly to multiple drug therapies are increasingly likely to suffer from multiple drug DHRs which are becoming an increasing clinical concern [13] due to their unpredictable nature as different multiple DHR can occur with each different drug regimen.

More than 95% of all DHRs are skin related reactions [14], with most of the reactions being classified as less severe; falling into the inconvenient but not necessarily dangerous category. It is believed that cutaneous DHRs are the more likely outcome due to the extensive network of blood vessels, which allow for T cells to quickly reach the site of action and cause some of the delayed type reactions observed. The less severe reactions observed are generally urticaria (hives) and other milder skin rashes which cause itching and mild discomfort. Stevens-Johnson syndrome and toxic epidermal necrolysis are two of the more severe cutaneous DHRs which are related to

skin detachment, leaving the patient open to infection. These reactions have a relatively high mortality rate; with high morbidity for those who are lucky enough to survive these reactions.

The time of onset of a DHR can help in some ways to classify the reaction, with reactions with an onset of less than one hour being qualified as immediate reactions mediated primarily by IgG antibodies and mast cells. Whereas delayed-type DHRs are reactions with an onset later than one hour after drug administration; but can last up to a month as they are T cell mediated which requires time for T cell priming and the initiation of an immune response [15].

1.2.1 CLASSIFICATION

The classification of DHR is somewhat separate to ADR classification; although there are links. The initial classification of DHR were created based on the time of onset of the reaction, as well as the perceived mediators of the reaction by Gell and Coombes in 1963. Instead of using an alphabetised classification system, Gell and Coombes decided that DHR would be categorised as **Type I-IV**.

Type I are immediate reactions, such as asthma or hay fever as well as anaphylaxis. They are mediated through the antigen cross-linking of immune cell receptors such as mast cells and bound IgE. Degranulation occurs and subsequent release of histamines and cytokines cause allergic responses.

Type II reactions are semi-delayed cytotoxic reactions involving natural killer T cells, neutrophils, eosinophils, macrophages as well as complement pathway activation through FC receptor activation of these immune cells downstream of IgG and IgM release from triggered B lymphocytes.

Type III reactions are also IgG, IgM linked semi-delayed reactions propagated through soluble antigen-antibody complexes in the blood. This causes complement pathway activation and the recruitment of macrophages and neutrophils. Lupus is one example of a type III reaction.

Type IV reactions are the major subset of DHRs of interest, especially in the development of *in vitro* T cell-based assays. They are delayed in onset and require antigen presenting cell-T cell interaction as a prerequisite. The classic model is one of an APC such as a dendritic cell presenting an antigen to T cells which become activated releasing inflammatory cytokines which cause the observable clinical response. DHRs such as Steven-Johnsons syndrome and toxic epidermal necrolysis can be categorized as a type IV response. Due to wide-spanning categorisation of DHR Type IV reactions, it was decided that it would be better to further categorise type IV reactions into 4 further subsets a, b, c and d, based on the immune cell type and cytokines involved in the reaction. Type IVa reactions involve macrophages and T cell IFN-γ release, IVb involve the release of interleukins (IL-) 4, 5 and 13 from T cells with eosinophil activation. IVc reactions are cytotoxic reactions mediated mainly by cluster of differentiation (CD) 4 and 8 cytotoxic T cells (CD4/CD8). Whilst IVd reactions involve neutrophils with the upregulation of GM-CSF and IL-8 release from T cells.

1.2.2 DIAGNOSIS

1.2.2.1 IN VIVO

One of the main ways in which DHRs is diagnosed is through the use of *in vivo* tests such as a skin prick, patch and intradermal tests. All of these tests have their limitations and issues associated with them, one of the main issues being the compliance of the

patient as they are invasive procedures that can lead to some discomfort; including some side effects. However, these skin-based tests were developed in response to immediate cutaneous reactions to β-lactam antibiotics such as amoxicillin [16,17]. Immediate IgE mediated drug eruptions can quickly be diagnosed by cutaneous tests like the prick/intradermal tests in less than one hour [18]. More recently *in vivo* tests have been used to diagnose reactions with a delayed onset. Tests when positive may take up to a few days to develop [19]. Of all the cutaneous hypersensitivity reaction *in vivo* tests, the most popular are the skin prick, skin patch and intradermal tests.

The Skin prick test involves placing a small amount of allergen onto the forearm of the patient, and then carefully scratching the outer layer of the skin in order to allow entry of the potential allergen. It has traditionally been used to test for immediate allergic reactions such as contact urticaria, asthma, and food allergy [20].

The Patch test is one in which the clinician attaches a patch containing the allergen to either the arm or the back, this is usually a skin allergen resulting in an immediate reaction [21]. This patch stays on for 48 hours in case of delayed onset DHR such as delayed onset urticaria, SJS and TEN [22].

The Intradermal test is one in which a small amount of the allergen is injected with a hypodermic needle under the dermis to determine immunogenicity of a drug to the patient. This has previously been used to determine allergy after anaphylactoid reactions to anaesthetics [23,24].

These *in vivo* tests are somewhat invasive and can lead to discomfort for the patient. In addition to this, these cutaneous tests have relatively low specificity, which is a trade-off in the interest of keeping the tests as safe as possible (sensitivity is the ability of a test to correctly identify those with disease, specificity is the ability of a test to

correctly identify those without disease). Amongst the above disadvantages, all the cutaneous DHR *in vivo* tests outlined above have the advantage of the possibility (however small) of inducing a response in a patient which may have harmful effects. The development of diagnostic and predictive *in vitro* assays will circumvent this issue, and potentially allow for more accurate, safer diagnosis and prediction of drug induced hypersensitivity reactions.

1.2.2.2 IN VITRO

1.2.2.2.1 PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood mononuclear cells (PBMC) must be obtained in order to carry out any of the following *in vitro* experiments as most of them rely on the availability of T cells, and in some instances, antigen presenting cells such as dendritic cells or monocytes. When PBMCs are isolated from patient samples, the availability of drug specific T lymphocytes are of the utmost importance, as their activation proves the diagnosis, and further work can be done to classify the phenotype of the reaction. Drug specific T cells are invaluable for the development of useful and accurate *in vitro* tests, and importantly are detectable in the PBMC up to several years after the final administration of the allergenic drug [25].

1.2.2.2.2 LYMPHOCYTE TRANSFORMATION TEST (LTT)

The Lymphocyte transformation test (LTT) is the most used *in vitro* assay for the diagnosis of DHR [26]. It makes use of PBMCs, which are cultured with the potential allergenic drug in various concentrations for 5 days. The readout of the assay which produces the result relies on the detection and quantification of T cell proliferation in response to the drug over the five day period, in which time the drug will have had time to be processed and presented by APCs such as dendritic cells, leading to T cell

activation and proliferation. Radioactive ^{3}[H] thymidine is added on day 5 which is incorporated into the DNA of the T cells, which can then be detected by a beta-counter. The detected T cell proliferation is compared to control samples without drug addition, and tetanus as a positive control (as most of the population will have been sensitised to tetanus at some point), the quantified T cell response can then be used primarily to determine if a DHR has occurred in response to the drug in question, but can also be used to assess optimal concentrations of drug to use. Some of the positive aspects of the assay are that it is less invasive than the use of *in vivo* cutaneous tests and has no chance of harming the patient in any way. PBMCs are readily available from blood and contain drug specific T cells which can be used in a plethora of ways in order to further understand DHRs. The LTT can also investigate both immediate and delayed onset DHR reactions, therefore nothing is lost when using the LTT in place of *in vivo* methods [27]. However, the use of radioactive ^{3}[H] thymidine to enable detection of lymphocyte proliferation increases the cost of the assay. At 6 days, the assay is also more time consuming and labour intensive than some of the older *in vivo* methods. Cross-reactivity between certain antibiotics can also be observed, leading to false positives in the assay [28], in addition to the fact that the assay cannot provide insight into the mechanism of the adverse event or immune aetiology of a DHR. Despite these shortcomings of the assay, the LTT remains as the most widely used *in vitro* assay in DHR diagnosis at this time.

1.2.2.2.3 Enzyme-linked Immunosorbent Spot Assay

An Enzyme-linked immunosorbent spot assay (ELISpot) is another useful diagnostic tool in drug hypersensitivity, as it gives more of an idea as to the phenotype of the reaction, rather than just the yes/no provided by the LTT. It works by pre-coating antibodies to a membrane in the well of a 96-well plate, and then culturing either

PBMCs or specific T cell populations with APCs and the drug in question. Upon activation of T cell by the drug, the cytokine to be detected may be secreted by the T cells which then bind to the specific antibodies on the pre-coated membranes. The cells are then washed off, and enzyme conjugated detection antibodies are added, followed by a substrate which causes the appearance of black spots on the membrane. Each spot is considered a site of cytokine secretion, meaning the more spots on the membrane, the more cytokine secretion is occurring. The ELISpot is both sensitive and specific; being able to quickly detect relatively low-level secretion of cytokines from T cells. The assay can be used to characterise the DHR by the detection of several cytokines secreted by activated T cells, including pro-inflammatory cytokines such as IFN-γ, anti-inflammatory cytokines such as IL-13, regulatory cytokines such as IL-10 and cytotoxic mediators like Granzyme B [29]. This information gives some insight into the type of reaction occurring, and the role of either a specific subset of T cells (if using T cell clones) or the type of response elicited by the total PBMC in response to drug challenge. For example, IFN-γ release from drug-specific T cells was detected in more than 90% of amoxicillin hypersensitive patients tested with ELISpot [30], and is able to achieve this more sensitively than the ELISA assay.

1.2.2.2.4 T Lymphocyte Cloning

Cloning of T lymphocytes is something to be discussed briefly, as although it is not strictly a diagnostic assay, it is an important mechanistic tool that can then be linked to a variety of other diagnostic *in vitro* techniques. T cell cloning is established through the culture of PBMC with drug for 2 weeks, at which time the cells are assayed into 96-well plates in 1 cell per well. The cells are then re-stimulated with irradiated heterologous PBMCs which stimulates extensive proliferation. Cells are kept alive and growing through repeated mitogen stimulation with IL-2 and can then be tested for

specificity by proliferation and ELISpot experiments. This protocol would strictly be classed as a mechanistic assay, as it allows for the clonal expansion of single drug-specific T cell populations. The further characterisation of these T cell populations then provides some mechanistic insight into reactions occurring, as well as the ability to find out a great deal about some of the T cells that are involved in DHRs. The T cell populations can be phenotyped for CD4/CD8, proliferation data can be acquired, as well as being able to profile the T cells by the types of cytokines they release either through the use of ELISpot or intracellular staining and analysis through flow cytometry. The use of T cell clones has helped to establish the characteristics of some T cell populations involved in DHR and can even help establish whether cross reactivity between antigens is occurring through the detection of one or more cell surface antigens. The use of this technique is important for the characterisation of the pathomechanisms involved in DHR and the detection of rare T cell populations in patients presenting with negative diagnostic assays [31,32]. However, T cell clones can be vulnerable to infections, exhaustion of loss of specificity if kept in culture for too long.

1.2.2.2.5 Flow Cytometry

Flow cytometry is one of the most widely used immunological techniques that can be used in an array of applications as it can be used for the detection and expression of cell surface markers, as well as for the detection and sorting of cell populations into specific subsets. Specifically, for DHR prediction and diagnosis immune cells can either be PBMC or tissue derived - with markers able to identify and sort specific immunological cell subsets, as well as the identification and expression of tell-tale immunological markers; including antigen-specific T cell subsets and their activation. Multiple markers can be concurrently analysed through use of fluorescently

conjugated antibodies, with their relative expressions compared vs unstained negative controls.

The CD69 upregulation assay makes use of the increased expression of the cell-surface CD69 marker following T cell activation. This can be detected by flow cytometry to determine the extent of T cell activation following antigenic stimulation [33–35].

The fluorescent biomarker carboxyfluorescin diacetate succinimidyl ester (CFSE) can be used to detect proliferative responses in T cells, as CFSE is incorporated into cells by interaction with intracellular proteins. Fluorescent intensity is halved by subsequent T cell divisions; which can be quantified by flow cytometry [36].

Flow cytometry can also be used to determine the presence of certain cell populations and their abundance within a culture in response to drug treatment; such as the increase in T regs observed during naïve T cell priming in Chapter 4 of this thesis.

Intracellular staining can also make use of fluorescent antibodies as well as flow cytometry to detect expression of cytokines and other cellular factors in response to drug treatment. The main difference between cell surface marker analysis and intracellular staining is the need to fix and permeabilise cells to detect intracellular expression. This can be used as an alternative to the ELISA and ELISpot assays and can be incorporated into other panels concurrently; an advantage over the ELISA and ELISpot systems.

1.3 THE IMMUNE SYSTEM

1.3.1 THE ROLE OF THE IMMUNE SYSTEM

The main role of the immune system is to protect the body against foreign antigens; including bacteria and viruses. It does this primarily through a variety of different immunological mechanisms that include variations on the detection, interaction with,

and ultimately the destruction of said foreign antigen. The innate immune system plays a major role in early immune defence mechanisms, whilst the adaptive immune system is often involved at a later point, including recognition of the same antigen upon re-exposure. This often concludes with the adaptive immune system being the main driving force of an immune mediated response to a specific antigen. However, the nature of the immune system means that there are milieu different immune mechanisms, and thus, many different ways in which the body reacts to protect itself upon antigenic recognition. Due to the extreme complexities involved in the mammalian immune system, the lines can be blurred between an innate and adaptive response. Immune homeostasis is critical for the healthy function of the human body, and any defects to the immune homeostasis can lead to the adverse events discussed throughout this thesis. Indeed, the innate immune system can react more generally to antigens in an immediate manner; however, once adaptive immunity has taken over, reactions are mounted against specific antigens in a more delayed manner.

The experiments contained within this thesis focus mainly on the adaptive immune system, as the experiments are designed to investigate delayed (type IV) DHR through the determination of outcomes of antigen-specific T cells upon exposure to the antigen; including the priming of naïve T cells and the related immune mechanisms.

1.3.2 LYMPHOCYTES

Lymphocytes, especially T cells (T lymphocytes), are central to the adaptive immune response. Naïve T cells can be polarised through exposure and interaction with cytokines into different categories of effector T cells, each with their own distinct role and mechanism of action. These include cytotoxic T cells (CD8+), helper T cells (CD4+), as well as regulatory T cells (Tregs) amongst others. T cells express a large range of receptors on their surface, most importantly, the T cell receptor (TCR) that is

required for interaction with the MHC of APC to form an immune synapse (signal 1). The ability of the TCR to recognise antigenic peptide fragments, displayed on the MHC of APCs is central to the formation of the immune synapse, and ultimately, T cell priming, activation, and clonal expansion. The mechanisms employed by differentiated T lymphocytes are mainly linked to the production of either pro- or anti-inflammatory cytokines dependent on the required outcome of action. Whilst B cells are generally differentiated into plasma cells that secrete antibodies in response to an antigen or are reserved as memory B cells.

Below is a summary of the main lymphocyte families involved in the immune response to an antigen and are therefore relevant to the study of DHR within this thesis.

1.3.2.1 CD8+ T CELLS

Cytotoxic T cells can be primed in the secondary lymphoid organs such as the spleen or lymph nodes from CD8+ naïve precursors in the presence of IL-7 [37]. CD8+ T cells are cytotoxic in nature and respond in an MHC class I restricted manner, meaning that the interaction of the CD8+ TCR is restricted to peptides displayed on the surface of MHC class I on APCs or other cell types that express MHC class I [38]. CD8+ T cells' mechanism of action is to target and destroy previously damaged or infected cells through the release of several cytotoxic and cytolytic molecules; including perforin, fas ligand (FasL), granulysin, and granzyme B. Granzyme B and granulysin work in tandem with perforin to elicit their killing effects on cells. Perforin forms pores into the targeted cells, enabling the entry of granzyme B and granulysin to cleave cellular proteins and induce cell death through caspase signalling dependent apoptosis. Granulysin can also cause membrane disruption to induce cell death. Both granulysin and granzyme B are important cytokines for their cytolytic killing effects, but are also important immunological cytokines that activate further downstream cytokine

signalling in an immune response, and the recruitment of other T cells and inflammatory agents [39,40]. FasL is another cytotoxic molecule released from activated CD8+ T cells and induces the caspase signalling pathway and subsequent apoptosis [41,42]. Due to the ability of perforin, granzyme B, granulysin and FasL to induce apoptosis and cell death, CD8+ cytotoxic T cells have been implicated in severe DHRs such as SJS/TEN. Keratinocyte destruction and subsequent skin detachment occurs as a consequence of cytotoxic T cell secretion of the aforementioned cytolytic molecules [43,44].

1.3.2.2 CD4+ T CELLS

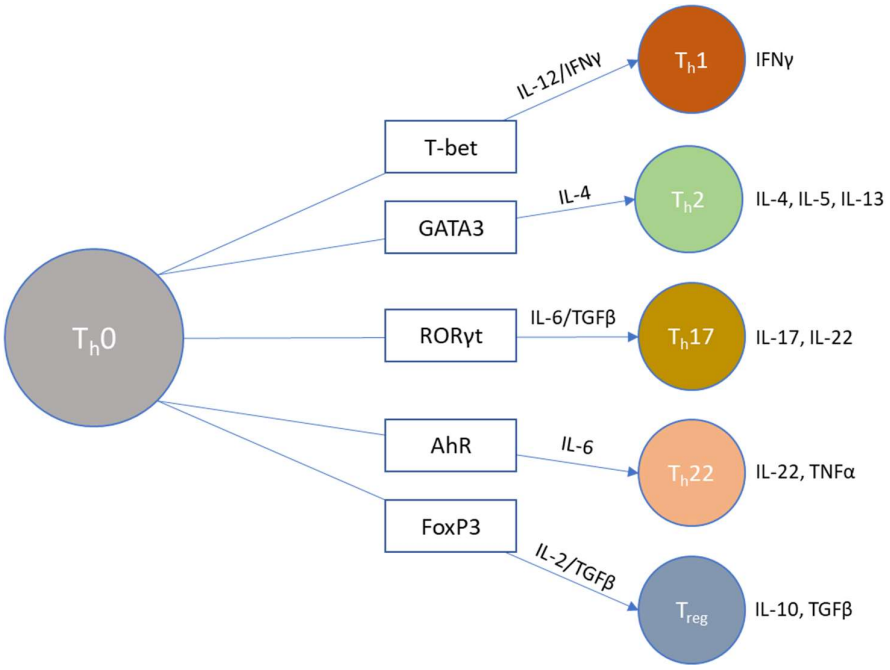


Figure 1.1 – T cell plasticity; differentiation of T_h0 naïve T cells to different subsets. Featuring the main transcription factor and cytokines influencing differentiation, as well as key cytokines secreted from each T cell type. Simplified and adapted from [45].

1.3.2.2.1 T HELPER CELLS

Naïve T Cells (T_h0) can differentiate into a number of different subclasses of lymphocytes dependent on the activated transcription factor. An important population

of T cells are T helper cells (primed from naïve CD4+ precursors), which can be further categorised into different subsets, identifiable by the transcription factor involved, the cytokines involved in the polarisation event, and the expression pattern of the cytokines they secrete. The main role of the T helper cell is the recruitment of other immune components to the site of infection/antigen detection through the secretion of specific cytokines dependent on the T helper subset involved. They are involved in the activation of cytotoxic T cells, as well as B cell class switching.

The best-established T helper subsets are the T_h1 and T_h2 T cells, as they were the first classes to be discovered experimentally. T_h1 cell development is driven by the T-bet transcription factor and IL-12 from the naïve T cell population, they are characterised by the secretion of pro-inflammatory cytokines such as IFN-γ. This secretion of IFN-γ can help protect against intracellular antigens (e.g. viruses) through pro-inflammatory activation of CD8+ cytotoxic T cells, macrophages and NK cells [46]. T_h2 cell development is driven by the GATA3 transcription factor, as well as the anti-inflammatory cytokine IL-4; which T_h2 cells also secrete for self-promotion and interaction with DCs. T_h2 cells were largely known as anti-inflammatory T helpers due to their secretion of IL-4, IL-5, IL-10 and IL-13. They promote B cell, eosinophil, and mast cell IgE mediated immunity against extracellular parasites and helminths [46,47]. Despite early research into T helper cells describing a T_h1/T_h2 balance in the adaptive immune system; many other cell types are also involved during a response. Indeed, in DHR patient studies, both IFN-γ and IL-13 have been detected, indicating that both T_h1 and T_h2 are involved in these iatrogenic responses [37]. Despite this, traditionally T_h1 cells were described as being involved in auto-immune diseases and Chron's, whilst T_h2 cells are involved in atopic disorders (presumably due to their IgE mediated action) [48].

More recently, the discovery of novel T helper subsets T_h17 and T_h22 have led to further implications of T helper involvement in drug allergy. T_h17 cells are driven by the RORγt transcription factor as well as IL-6 and TGF-β, whereas T_h22 cells require AhR transcription factor and IL-6 alone. T_h17 cells are known to secrete both IL-17 and IL-22, whilst T_h22 cells secrete IL-22 and TNF-α. T_h22 cells are involved in skin inflammation and DHR such as reactions to piperacillin treatment; IL-17 augments inflammation, IL-22 infers a protective role on the tissue [49,50]. Furthermore, work within the group previously detected IL-22 secreting antigen-specific T cells during naïve T cell priming assays to piperacillin when PD-1 signalling was blocked [50].

Less studied T helper subsets such as T_h9 (IL-9 secreting, PU.1 transcription factor driven) have been implicated in both IBD and skin disorders [51,52]. T follicular helper (Tfh) cells driven by Bcl-6 are located within temporary germinal centres and interact with B cells through a CD40 co-stimulatory pathway (to form IgE responses), and also secrete IL-21 and IL-4. They have been a target of interest for improvement of vaccination strategy, but have also been implicated in auto-immunity [53,54].

1.3.2.2.2 T Regulatory Cells

Tregs are characterised by an expression profile of CD4+CD25+FOXP3+ and are an important cell type involved in maintenance of immune homeostasis through immune regulation [55]. They are differentiated from naïve CD4+ T cells as part of T cell plasticity through the forkhead box protein 3 (FOXP3) transcription factor and are further influenced through TGF-β signalling [56,57]. They are an important immune self-check mechanism as they can establish tolerance against self-antigens or be the determining factor in whether an appropriate immune response is mounted against a foreign antigen. Tregs are known mainly for their immuno-suppressive actions which can be implemented via different mechanisms. Tregs express high levels of CTLA-4

(discussed in section 1.5.1), leading to decreased Treg-APC interaction; causing lymphoproliferative suppression as a viable immune synapse cannot be formed [58,59]. Tregs have also been implicated in IL-2 uptake – a key interleukin involved in T cell activation – which limits availability to, and subsequent activation of other T cells. This is also important as IL-2 has also been shown to induce Treg development and so despite its role in T cell proliferation activation of effector T cells, can also induce immunosuppressive events [60]. The secretion of the anti-inflammatory cytokines IL-10 and TGF-β from Tregs also form a large part of their immunosuppressive role in the immune system; again, through presumed disruption of APC antigen presentation to circulating TCR [61]. The importance of Tregs on immune homeostasis is outlined by the fact that defective Tregs have been found to be accountable for the exacerbated immune responses observed in DHRs such as hypersensitivity pneumonitis [62].

1.3.2.3 B CELLS

B lymphocytes developed in the germinal centres and are known to express CD19 and are involved in antibody production in response to antigenic recognition by various immunoglobulins expressed on their cell surface. CD19 acts in a synergistic manner with cell surface immunoglobulins to induce B cell maturation after peptide recognition on MHC class II of T cells [63]. B cells can be differentiated into antibody secreting effector plasma cells, or long-lived memory B cells through the cross-linking of their surface receptors. Plasma cells utilise complement pathway fixation and antibody secretion for the removal of viral fragments and can undergo class switching through somatic hypermutation at germinal centres which regulates the nature of the antibodies produced (IgM, IgE, IgG, IgA). Whilst memory B cells are long-lived and may remain dormant until re-exposure to an antigen, at which point polyclonal antibody production mediated by IgG and IgA immunoglobulins occur. Antibodies

produced by B cells can either become solubilised within the blood plasma (forming part of humoral immunity), or remain bound to B cell receptors on the cell surface [64].

Antibodies secreted by B cells can take part in adverse events such as tissue injury; and are also involved in autoimmune pathogenesis through their interaction with T cells including antigen presentation, cytokine secretion, and co-signalling pathway activity [65].

B cells were used experimentally within this thesis as an APC in chapter 6 – immortalised EBV transformed B cells were developed due to their ease of long-term culture, their antigen presenting capabilities, and their ability to be irradiated (preventing proliferation during antigen-specific T cell proliferation assays).

1.3.2.4 Natural Killer Cells

Unlike T helper cells, natural killer (NK) cells are effector T cells involved in innate immunity through secretion of cellular damaging molecules (similar to cytotoxic T cells). However, they can also form interactions with DCs, macrophages, and other T cells to elicit a regulatory phenotype under certain circumstances [66]. They are deemed part of the innate immune system as they do not use TCRs to respond to specific antigen like members of the adaptive immune system (T cells). They respond in a more general manner; using Ly49 and killer Ig-like receptor (KIGR) to carry out anti-tumour, and anti-viral responses and maintain tolerance through the distinction between self and non-self. NK cells use KIGR to detect down-regulation of MHC class I in circulating cells which acts as a targeting mechanism for NK cells, as MHC class I is down-regulated during cellular stress/damage. NK cells respond to a multitude of cytokines including pro and anti-inflammatory: IL-12, IL-18, IL-27, IL-35, IL-2, IL-7 and IL-15 [67,68]. NK cells also secrete cytolytic molecules such as granulysin and granzyme B to induce apoptosis in targeted cells in an immediate innate manner.

However, activation of TLRs on NK cells can lead to IFN secretion, which in turn can induce T-bet transcription factor and the subsequent activation of the adaptive immune system through T_h1 responses [66,69,70].

1.3.3 MONOCYTES – MACROPHAGES & DENDRITIC CELLS

Monocytes are differentiated from a precursor myeloid cell progenitor cell; they have a single nucleus, and overall are larger than other granulocytes. They can be characterised by their expression of the cell-surface marker CD14, something which we select for when separating monocytes from whole PBMC [71]. Monocytes are then able to differentiate into further subpopulations of monocytic phagocytes; macrophages (M1 or M2) or DCs, dependent on the surrounding cytokine signalling microenvironment, or alternatively remain as inflammatory Ly6C+ monocytes.

M-CSF as well as IL-6 influences the differentiation pathway towards macrophages, whose job is the recognition (through constitutively expressed TLRs) and phagocytosis and degradation of antigen through lysosome fusion and ROS/nitric oxide destruction. This is in addition to their role in antigen presentation to circulating T_h1/T_h2 cells and the recruitment of granulocytes such as neutrophils [72,73]. Macrophages can be classed as either M1 macrophages which secrete pro-inflammatory cytokines such as IFN-γ and induce a T_h1 mediated response, or M2 macrophages which are considered anti-inflammatory; resulting in a largely T_h2 response [74].

Dendritic cells are the main APC used experimentally within this thesis and are the one of the main initiators of the adaptive immune response *in vivo*. Experimentally, we were able to differentiate CD14+ monocytes to DCs through a 7-day culture with GM-CSF and IL-4 as well as TNF-α stimulation [71]. *In vivo*, TNF-α is a major differentiating factor in DC development as it limits the M-CSF pathway which would

usually result in macrophage development. DCs are one of the most important barriers to whether or not adaptive immune activation occurs due to their involvement in recognition of foreign antigen by TLRs, engulfment and internalisation of the antigen, internal processing of the antigen to antigenic peptides, before presentation to circulating T cells on the upregulated MHC class I or II. DCs are found in the peripheral blood but migrate to secondary lymphoid organs upon maturity to elicit their professional APC capabilities and form the backbone of adaptive immunity and immunological memory. During immaturity dendritic cells function better as phagocytes, acting to engulf and internalise foreign antigen – whereas upon maturity they become larger and more granular, forming their trademark dendrites (see *figure 1.2)*. This along with the upregulation of MHC class II allows for better antigen presenting capability and the formation of an immune synapse (signal 1). DCs are further involved in T cell mediated responses as they also up-regulate both CD80 and CD86 - involved in the co-stimulatory CD28 family pathway (signal 2) – as well as the co-stimulatory CD40. Both signal 1 and signal 2 are a pre-requisite for adaptive immune activation (section 1.5.1) [75,76]. Maturity of dendritic cells can be induced by a number of factors interacting with TLRs on the cell surface, these include: activation by damage associated molecular patterns (DAMPS) such as HMGB1, or heat-shock proteins such as HSP70 [77,78]. Pathogen associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS) are also used experimentally to induce DC maturation *in vitro* [71,79]. DC maturation can also be directly induced by drugs or CRMs such as our major experimental compound SMX-NO; potentially acting through a DAMPS mechanism through cell toxicity [80]. It is for the above reasons DCs play such a vital role in the adaptive immune system and is the ideal candidate to be used as the predominant professional APC in experiments throughout this thesis.

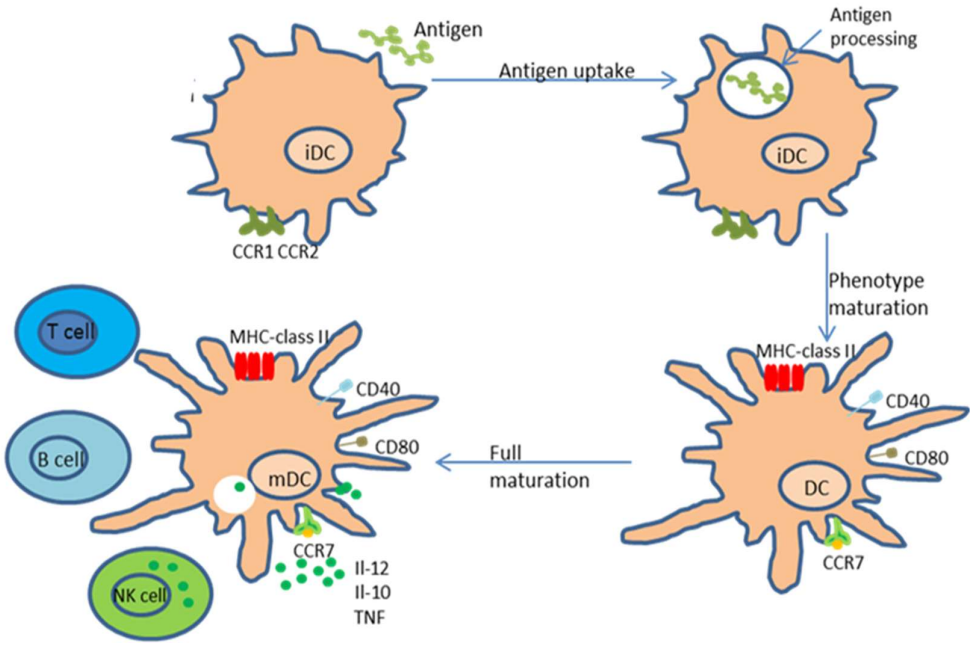


Figure 1.2 – The maturation of dendritic cells. Simplified and adapted from [81].

1.3.4 Other Immune Components - Granulocytes

There are also a range of other cell types involved in the mediation of innate immunity. These are not involved in experiments contained in this thesis but are important for immediate DHR. They are renowned for their multi-lobed nucleus and originate from myeloid precursors.

Neutrophils are the most abundant granulocyte and are involved in the primary immune response through chemotaxis to the site of inflammation. Once there they identify the target, before engulfment and phagocytosis, followed by destruction internally by ROS, lysozymes and cathepsins [82]. Neutropenia – low concentration of neutrophils in the blood – can be a serious detriment to the primary immune response in an individual and can be caused due to systematic diseases leading to congenital disorders, or through self-limiting viral infection [83].

Basophils are the least common granulocyte. IgE mediated activation leading to degranulation results in the release of multiple signalling molecules such as histamines heparin and proteolytic enzymes, as well as DC differentiation factors GM-CSF and IL-4. They are also known to be involved in T_h2 mediation [84].

Eosinophils are another granulocyte that are derived from progenitor stem cells before circulation in the blood. Eotaxins and IL-5 signalling are involved in the recruitment of this cell type to inflammatory sites, where a range of pro-inflammatory cytokines, cytotoxic granule proteins, and ROS are released [85]. Severe inflammation can occur when the concentration of eosinophils within the blood becomes abnormally elevated (450-550 cells/uL of blood) – this is known as eosinophilia [86].

Mast cells are also involved in innate immunity – they are a subset of granulocytes derived from myeloid precursors. They are larger granular immune cells secreting many of the same inflammatory molecules as basophils. Upon activation through B cell secreted IgE cross-linking, de-granulation occurs with the previously mentioned molecular inflammatory mediators being secreted. Mast cells are involved in many IgE-mediated allergic reactions and participate in failure of immune tolerance which may lead to autoimmunity [87,88]. Severe allergic responses can be observed when mastocytosis occurs; an excess of mast cells locating in the tissues.

1.4 T CELL ACTIVATION & GENETICS

The activation of T cells *in vivo* is largely a function of the interaction between an antigen, the human leukocyte antigen expressed on antigen presenting cells (APC), and the T cell receptor. The interaction between these three immunological components can lead to the formation of an immune synapse, and thus completes at least one stage of the necessary interactions leading to the initiation of T cell response. Due to the major role inferred by the HLA on immune activation, there is a heavy

genetic component involved in this process. Genetic associations with disease were first recognized in the early 20th century, when Archibald Garrod discovered that alkaptonuria – a disease which prevents the processing of phenylalanine and tyrosine by the body – followed Mendel's rules of inheritance and relied on a recessive mutation [89,90]. Since then, other diseases have been revealed to have a genetic aetiology, leading to generational inheritance. Variations in the population's genetics mean that some people are predisposed to developing illnesses such as specific cancers, or hereditary conditions like cystic fibrosis or Huntington's disease. In some instances, the severity of the symptoms can be linked to distinct genetic mutations in the individual.

1.4.1 HUMAN LEUKOCYTE ANTIGEN

The HLA gene complex encodes a multitude of proteins that are largely responsible for the body's ability to mount an adaptive immune response to an antigen. First identified in the 1950s, it took 30 years before the structure and function of the HLA was elucidated through seminal work on the HLA-A*02 allele by Bjorkman and Wiley [91]. HLA alleles are split into Class I and II, which encode HLA class I and II proteins, respectively. Variations in the sequence of the HLA peptide-binding groove are responsible for intraindividual variations in the body's response to antigens (including drugs). Due to the highly polymorphic nature of the HLA gene complex, there are allelic variations of both classes. HLA-A, HLA-B, and HLA-C are individual loci of HLA class I proteins, with each locus possessing multiple alleles, whereas HLA-DR, HLA-DP, and HLA-DQ are HLA class II variants [92–94]. There are several thousand allelic variants of HLA class I and II that encode proteins with differing structures that in theory will present different peptides on the surface of APC. Presentation allows for the recognition of an antigen by a T cell receptor and is necessary for the formation

of an immunological synapse and T cell activation. The subdivision into HLA classes I and II can be determined both by structure and the nature of antigen binding. HLA class I proteins are expressed on all nucleated cells and present peptides of intracellular origin to CD8+ T cells. In contrast, HLA class II expression is mainly restricted to APC and the HLA proteins present peptides derived from extracellular proteins to CD4+ T cells. HLA loci are the most polymorphic of the human genome leading to a broad range of genetic diversity. The nomenclature of HLA helps to distinguish and categorize the differences between the different proteins (*Figure 1.3*). Although the structure of HLA class I and II proteins are different, they are both are heterodimeric (*Figure 1.3*).

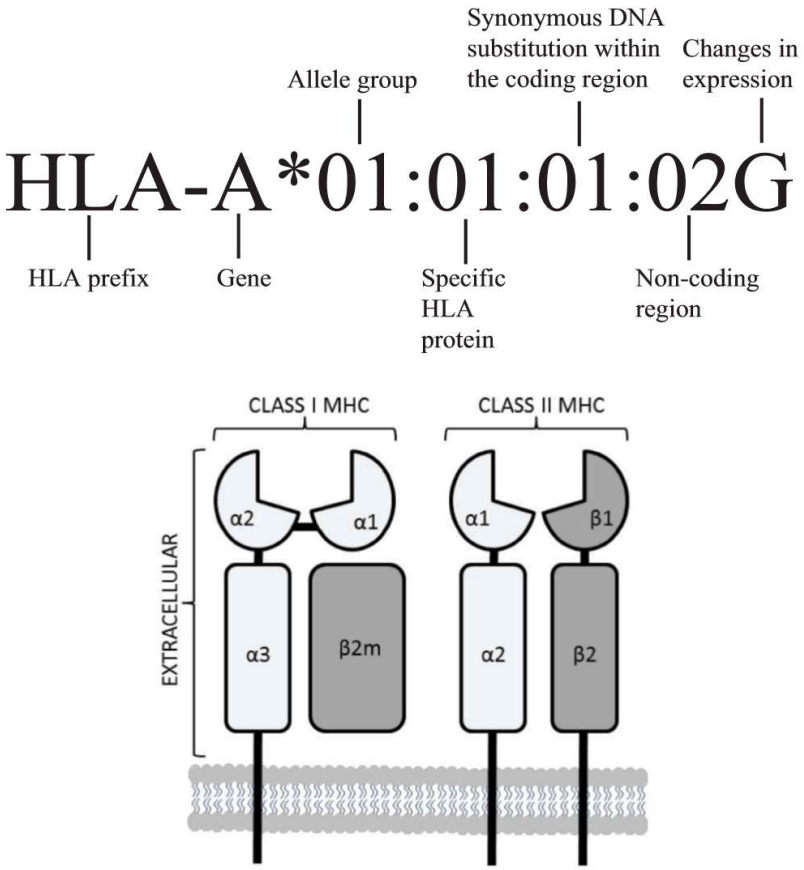


Figure 1.3 – Nomenclature and structure of HLA class I and II molecules [95].

HLA class I consists of an alpha chain encoded by the HLA class I gene, which, makes up the peptide-binding groove that interacts with β-2 microglobulin. HLA class II proteins are composed of an alpha and a beta chain that are encoded by individual HLA class II A and B gene loci, respectively. Different ethnic populations express a range of common alleles and this has resulted in several forms of DHR primarily affecting specific geographical regions. For example: (1) carbamazepine-induced Stevens–Johnson syndrome is strongly associated with HLA-B*15:02; and (2) dapsone hypersensitivity syndrome is strongly associated with HLA-B*13:01. Both of these alleles are expressed at high levels in Chinese populations but are almost absent from Caucasians. This topic is covered in more detail below. The identification of HLA allele associations with DHRs has allowed researchers to focus on specific drug interactions with single HLA proteins. The three most studied examples being HLA-B*57:01 with abacavir and flucloxacillin and HLA-B*15:02 with carbamazepine. At the turn of the century, drugs were thought to interact with HLA via two basic mechanisms: either the hapten or pharmacological interaction (p-i) pathway. After 15 years of intense research, the importance of these pathways to different forms of DHR is now clearer. Furthermore, studies focusing exclusively on abacavir have identified a third alternative or complementary pathway coined the altered peptide repertoire concept. Each of these pathways are discussed below and then specific well-defined examples will be used to describe how our knowledge of each pathway has progressed. Central to the understanding of drug action is the way in which a drug binds to its target to elicit a wanted or unwanted response. As Paul Ehrlich stated over a century ago, ‘a drug will not work unless it is bound’. In terms of DHRs, a drug must somehow bind to HLA proteins (or an HLA binding peptide) and interact with specific T cells. Whether a T cell response is induced is determined

by the specificity of the binding interaction, the site of binding within the receptor, the complex nature of the bound drug, and finally the extent of binding. A plethora of specific receptors exist so that drugs cannot bind promiscuously, providing a self-regulation checkpoint against continually causing unwanted adverse reactions. Furthermore, the interaction between drug and HLA proteins does not guarantee triggering activation of an immune response. For drugs that induce DHRs, two things must occur. First, the T cell receptor must be bound by an HLA-associated drug, and second, the activation threshold of the T cell receptor must be reached for the drug to act as an immunogen and initiate an immune response [96–98].

1.4.2 Models of Drug Hypersensitivity – Binding Mechanisms of HLA and TCR

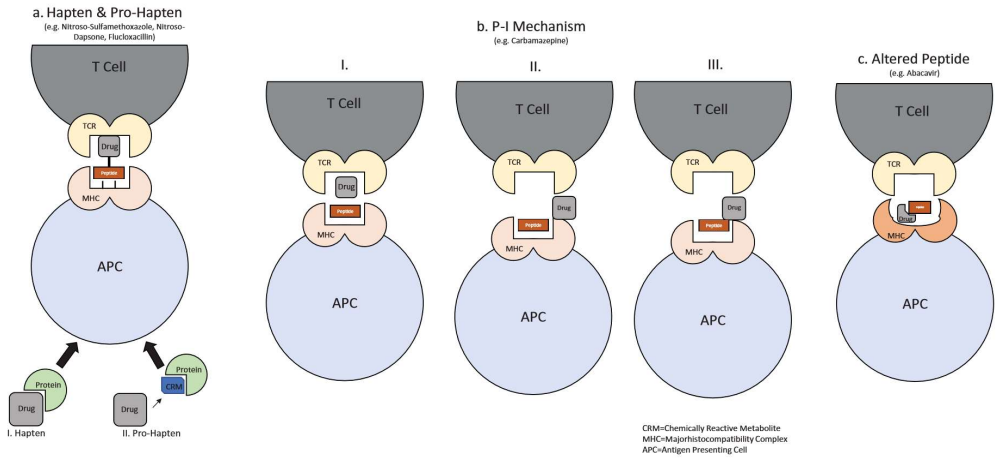


Figure 1.4 - Different mechanisms of T cell activation by drugs. A. Haptenation occurs when a drug hapten binds to a protein to form an adduct. The protein adduct can then be internalized and processed by antigen presenting cells into peptides for loading onto the HLA, being presented to circulating T cells. The pro-hapten theory differs solely in the fact that the drug first must form a chemically reactive metabolite before binding to the protein to form the adduct. B. In the p-i pathway, the drug can interact directly and non-covalently with the HLA-TCR complex to induce an immune response. The nature of this binding interaction has not been defined. No internalization or processing by the antigen presenting cell is required. C. The altered peptide concept is similar to p-i in that it is a non-covalent interaction. However, antigen presenting cell processing is required to generate peptide sequences that bind to the abacavir-modified HLA. The altered repertoire of self-peptides may then induce an immune response [99].

1.4.2.1 THE HAPTEN HYPOTHESIS

The interaction between HLA located on specific antigen-presenting cells, peptide and the T cell receptor is a critical initiator of the T cell response. This interaction is often termed signal 1. The hapten hypothesis – first proposed in 1935 by Landsteiner and Jacobs' working on small chemically reactive compounds – states that a chemical (or drug) must bind covalently to a protein to induce an immune response [100]. Studies performed in subsequent decades have confirmed the original hypothesis and shown that lymphocyte reactivity with small chemicals is directly related to protein reactivity [101–105]. Work into the penicillins showed that lysine residues were covalently bound by beta lactam antibiotics; these modified penicillin protein haptens were later determined to be able to activate T cells [106,107]. Soon after this synthetic peptides were used to explore this phenomenon, with penicillin-modified peptides being found responsible for T cell activation [108]. Work into SMX also helped to confirm the hapten concept and the involvement of chemically reactive metabolites in T cell activation. SMX was found to form a reactive metabolite (SMX-NO) via the intermediate hydroxylamine (SMX-NHOH)[109]; the reactive metabolite was later found to drive T cell response in animal models [110]. SMX-NO specific reactive T cells are known to be dependent on antigen processing, as pulsing APCs with SMX-NO (washing free drug away) leads to T cell activation. No response is observed when APCs are fixed to block antigen uptake [111].

The term hapten refers to the chemical entity that binds covalently to protein, whereas an antigen is the HLA-binding peptide derived from the protein adduct after processing by antigen-presenting cells. We tend to assume that the drug molecule remains attached to the HLA-binding peptide to activate T cells; however, this assumption could be wrong as drug binding may alter protein processing. New peptide

sequences might be generated, and these could interact with HLA proteins and trigger T cell receptors. The reason why the study of drug hapten T cell responses is so intriguing and so complicated is that virtually nothing is known about the drug modified proteins involved in the immune response. Moreover, most drugs are inert on administration and undergo metabolism before they form a drug-protein complex. Drugs that activate T cells via a reactive metabolite are referred to as pro-haptens [112–114]. Since metabolites presumed to be involved in DHRs are intrinsically unstable, very few are available for functional studies. As such, our understanding of the mechanistic basis of drug-specific T cell activation is highly skewed. The vast majority of experiments conducted to date involve parent drugs that do not form relevant metabolites in immune cell cultures. Thus, it is highly unlikely that a drug-metabolite-induced response will be detected in these assays.

1.4.2.2 The Pharmacological Interactions (P-I) Concept

We now know that an immunological synapse can also form through a drug interacting directly via noncovalent bonds with HLA peptide and the T cell receptor. This is referred to as the p-i concept. The concept originates from the observation that the lymphocyte transformation test – a diagnostic assay based on the proliferation of peripheral blood mononuclear cells (PBMC) from patients with DHR, but not tolerant controls, is positive when the cells are incubated with the parent drug [115,116]. Furthermore, experiments using drug-specific T cell clones have shown that a hapten pathway for T cell activation in certain instances is not possible. First, antigen presenting cell fixation, which blocks antigen processing, does not inhibit the T cell response observed with certain drugs. A hapten pathway of T cell activation is dependent on protein processing; thus, T cells must be activated via a different mechanism [117]. Second, drug antigen-presenting cell pulsing experiments, where free

drug is washed away, prior to exposure of the antigen-presenting cells to T cells yields negative responses [118]. Thus, the T cells seem to be activated through a labile readily reversible (non-covalent) interaction with antigen-presenting cells. Finally, researchers have shown that the kinetics of T cell activation are too quick for antigen processing to occur [119]. Several drugs including sulfamethoxazole, carbamazepine, lidocaine, and lamotrigine have been reported to directly activate PBMC and cloned T cells from patients with DHRs [119–122]. Although few now doubt that T cells are activated with drugs bound directly to HLA molecules, several important questions still need to be addressed. In particular, research is needed to define the site of drug HLA binding (peptide binding cleft, peptide, outside the cleft), the specific amino acids involved in the binding interaction and whether p-i activating drugs prime naïve T cells.

1.4.2.3 The Altered Peptide Repertoire concept

Researchers working on cloned T cells found that abacavir was able to activate certain cells rapidly – presumably via the p-i pathway; however, other clones were activated via a very different mechanism. With these clones, the response was dependent on protein processing. Furthermore, antigen-presenting cells pulsed with abacavir for 16 h stimulated IFN-γ release from the clones [123,124]. Antigen-presenting cell pulsing assays are normally used to show that drug protein adducts stimulate T cells via a hapten mechanism [117]. Originally, these data lead researchers to propose a hapten mechanism for abacavir-specific T cell activation; however, time-course pulsing experiments yielded unexpected results. Clones were not activated with antigen-presenting cells pulsed with the drug for shorter time periods; such as 1–4 h. What we now know is that abacavir needs to be present while proteins are being processed to liberate new HLA-binding peptides. The drug binds deep in the HLA protein binding

pocket altering the cleft structure and hence the peptide sequences that bind. These data led to evolution of the altered HLA peptide repertoire-binding concept – a second pathway of T cell activation occurring through noncovalent interactions between drug, HLA, HLA-binding peptide, and the T cell receptor. The concept evolved in 2012 when four independent research groups identified that the pathway of HLA binding could not be fully rationalized in terms of the hapten or p-i concepts [123,125–127]. What the altered peptide concept proposes is that T cells are actually activated with novel abacavir-dependent peptide sequences and that the drug is unlikely to participate in the T cell receptor binding interaction. In 2012, the data were so revolutionary because abacavir hypersensitivity is only detected in patients expressing one HLA allele – B*57:01 [8,128] and hence the drug must display exquisite sensitivity for this receptor. However, to date, abacavir is the only that selectively activates CD8+ T cells. This is in stark contrast to all other drugs associated with DHRs where drug-specific CD4+ and CD8+ T cell responses are seen. Based on this observation alone, it is not so surprising that abacavir activates T cells via a unique pathway. It must be noted that the formation of an immunological synapse is not necessarily sufficient in order for a T cell reaction to occur, as a T cell can undergo apoptosis and elimination when its cognate T cell receptor undergoes binding in the absence of activating cytokines or co-stimulation (signal 2). The presence of signal 2, as well as activation of APCs through danger signalling from damaged host cells may be required for an immune response to occur upon formation of an immunological synapse between drug and cognate T cell receptor.

1.4.3 MAJOR DHR ASSOCIATIONS

Investigation of drug interactions with HLA proteins is now heavily directed by pharmacogenomic studies identifying an increasing number of associations between HLA alleles and specific forms of DHR, and this topic is discussed below.

1.4.3.1 ABACAVIR

The most widely researched link between HLA risk alleles and DHRs is abacavir and HLA-B*57:01. Though abacavir is an effective component of combination therapy for patients with HIV, instances of DHRs after abacavir treatment were reported to have a prevalence of approximately 8% [7]. Abacavir hypersensitivity presents with multiple symptoms, including abdominal pain, fever, headaches, nausea, respiratory problems, including skin rashes and gastrointestinal symptoms [128]. In 2002, genetic studies identified that variation in the polymorphic HLA-B region played a role in the development of abacavir hypersensitivity; almost 50% of a group of 84 patients expressed the HLA-B*57:01 allele, whilst less than 5% of the control group had the allele [129]. In the same year, a second study identified the same HLA-B allele as a contributing factor alongside the class II alleles HLA-DR7 and HLA-DQ3. Although we now believe the class II alleles are not involved in the pathogenesis of the DHR, these early studies suggested that abacavir withdrawal from patients who expressed the risk alleles would reduce the prevalence of the DHR from 9% to 2.5% [128]. Subsequently, the Predict-1 study, which combined prospective HLA-B*57:01 screening with patch testing to confirm diagnosis, went on to demonstrate that screening eliminated abacavir hypersensitivity [8]. As discussed above, the altered peptide model has been proposed to explain how abacavir activates T cells. Knowledge that HLA-B*57:01 expression was critical for the development of the reaction allowed researchers to generate an X-ray crystal structure of the HLA

abacavir peptide complex and hence identify the important amino acid binding sites and most importantly show that the drug binds to HLA in a deep pocket under the peptide [126,127,130]. The model proposes that drug induced changes in the chemistry and structure of HLA-B*57:01 alters the self-peptides that bind, allowing for the recognition by circulating T cells of normally harmless peptides. The structure of abacavir is such that the compound only binds to the protein encoded by the HLA-B*57:01 allele; binding does not occur even with proteins encoded by closely related alleles such as HLA-B*58:01. Based on this data, we have recently combined computer modelling of the abacavir HLA-B*57:01 binding interaction with assessment of CD8+ T cell responses and antiviral activity using a panel of abacavir analogs to identify a safer abacavir derivative [130]. Each compound was synthesized with a substitution at the 6-amino position. The study demonstrated that it was possible to remove the unwanted HLA-B*57:01 binding of abacavir and T cell activation, whilst retaining some antiviral activity. As such it may be possible to design safer antiviral agents that do not require a personalized approach to therapy. As research continues, it is critical to identify whether the altered peptide repertoire concept does indeed apply only to abacavir. Moreover, it is essential that the nature and origin of T cell stimulatory peptides are elucidated. Currently, it is not known whether 'abacavir-responsive' T cells are activated by a single peptide or a multitude of peptides originating from different protein sources. It is intriguing to also consider whether T cell stimulatory peptides derive from endogenous peptides not normally seen by the immune system or peptides derived from microbes such as viruses. In this respect, White et al. [131] provided a detailed and thought-provoking insight into the concept of heterologous immunity, which in very simple terms suggests that patients induce a stepwise immune response first to virus and secondly to a drug such as abacavir in this

instance. The virus-specific T cells then recognize endogenous peptide presented in the context of HLA and drug to bring about the DHR. In theory, this concept could be investigated by generating virus-specific T cells from patients with and without a DHR and then exploring cross-reactivity with drugs. This would be of great use but may be made more complex due to the highly diverse nature of both the human virus, and the generated T cell receptors.

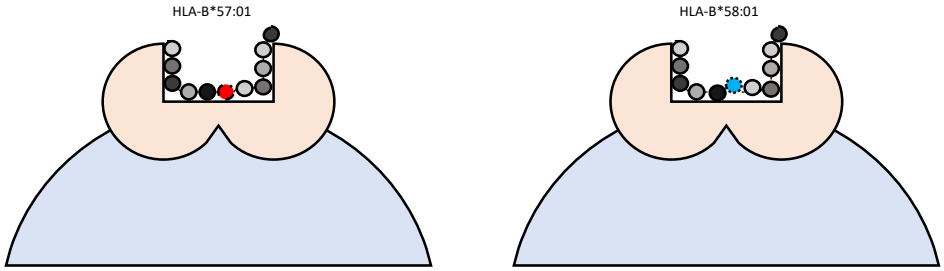


Figure 1.5. HLA-B*57:01 differs from HLA-B*58:01 in the peptide binding groove by only 1 amino acid residue. This affects what can interact with the binding groove, inducing immune responses. Some drugs interact with the HLA more specifically than others, for example people with the HLA-B*57:01 allele are known to be pre-disposed to abacavir induced DHR. This is not seen in people with the HLA-B*58:01 allele, whereas flucloxacillin induced DHR has been observed in patients possessing either of these alleles. Abacavir can only interact specifically with HLA-B*57:01, whilst flucloxacillin acts more promiscuously in nature, interacting with HLA of the same serological family particularly ones with very close amino acid residue sequences.

1.4.3.2 SULFAMETHOXAZOLE AND DAPSONE – REACTIVE METABOLITES

The sulfonamide antibiotic sulfamethoxazole is the drug that has been used most extensively to explore the role of metabolism in DHRs. This is primarily because the reactive nitroso metabolite of sulfamethoxazole has been synthesized [109] and can be used in functional assays. CD4+ and CD8+ T cells from all hypersensitive patients studied to date are activated with the parent drug and the nitroso metabolite [117,132,133]. Certain clones are activated selectively with the parent drug via a p-i pathway, whereas others are activated with nitroso sulfamethoxazole via a hapten mechanism. The picture becomes more complicated than this however as other clones are cross-reactive

and activated with both the parent drug and metabolite via different pathways. Furthermore, nitroso sulfamethoxazole can stimulate T cells through the direct covalent modification of HLA molecules and/or embedded HLA binding peptides. Despite these data sulfamethoxazole hypersensitivity reactions are not strongly associated with a single HLA allele [134,135]. The absence of an HLA risk allele in sulfamethoxazole hypersensitivity may explain why it is possible to prime naïve T cells to nitroso sulfamethoxazole in almost 100% of healthy donors [71,136,137]. T cell receptors containing the Vβ20-1 have previously been implicated in hypersensitivity reactions to drugs. Modelling data suggest that the CDR2β domain of the Vβ20-1 T cell receptor docks sulfamethoxazole with high affinity, and furthermore affects T cell receptor sulfamethoxazole-binding affinity through its action as a ligand controlled switch when SMX modifies three CDR2β amino acid residues: TYRβ57, ASPβ64, and LYSβ65 [138]. However, we are yet to fully understand how this modelling data translate into the activation of T cells with sulfamethoxazole. In contrast to sulfamethoxazole, dapsone hypersensitivity among patients with leprosy is strongly associated with HLA-B*13:01 [139]. In fact, the presence of HLA-B*13:01 had a sensitivity of 85.5% and a specificity of 85.7% as a predictor of the DHR. HLA-B*13:01 is present in 2–20% of Chinese people but is largely absent from Caucasians and Africans. Importantly, genetic screening for expression of the HLA allele has recently been shown to have significant utility as a genetic marker of the DHR [140]. Modelling data have shown docking of dapsone bound to HLA-B*13:01; and has outlined that binding affinity of dapsone to HLA-B*13:01 should be greater than to HLA-B*13:02 according to free energy calculations, and difference in F pocket structure between the two HLA alleles [141]. Dapsone is metabolized to a nitroso metabolite that binds covalently to cellular proteins [142,143]. For this reason, dapsone

hypersensitivity represents the ideal model to explore drug and drug metabolite binding to an HLA risk allele (i.e. HLA-B*13:01) and the pathways of drug presentation to T cells in patients with hypersensitivity. For this reason, we have recently synthesized nitroso dapsone and developed cell culture methods to study the priming of naïve T cells with the parent drug and metabolite. As both compounds activated naïve T cells via different pathways, similar to sulfamethoxazole and its nitroso metabolite, in HLA-B*13:01 negative donors [139,144], we are now beginning to explore whether the parent drug or drug (metabolite) peptide adducts preferentially bind to HLA-B*13:01 to activate CD8+ T cells from patients with hypersensitivity. Sulfamethoxazole contain an aromatic amine linked to a sulfonamide group. Similarly, nonsteroidal anti-inflammatory drugs such as celecoxib contain an aromatic sulfonamide moiety. Both drugs cause a relatively high number of hypersensitivity reactions, but the adverse events are seemingly not linked to expression of HLA-B*13:01. Thus, it is possible that the sulfone component and not the aromatic amine of dapsone in some way contributes to the selective interaction of the drug with HLA-B*13:01.

1.4.3.3 FLUCLOXACILLIN

Advances in our understanding of the role HLA alleles from diverse ethnic backgrounds play in DHRs has led to implementation of screening of patients from relevant ethnicities to abrogate reactions to abacavir, carbamazepine, dapsone, and allopurinol. Though these examples are considered as ‘success stories’ of genetic screening, most other HLA associations are not strong enough to warrant the screening or removal of an otherwise useful drug. One such example is flucloxacillin, a β-lactam antibiotic. Flucloxacillin is used in the treatment of gram-positive bacterial infections, but its use is associated with a high incidence of liver injury [145,146] and several deaths

have been reported. Flucloxacillin-induced liver injury is associated with expression of HLA-B*57:01. Although 43 out of 51 liver injury cases in the initial cohort expressed the risk allele, only approximately 1 in 1000 HLA-B*57:01 carriers go on to develop liver injury when exposed to flucloxacillin. Therefore, to prevent 1 incidence of flucloxacillin-related liver injury, greater than 13,000 individuals would need to be screened [145,147,148]. For this reason, HLA-B*57:01 screening will not be introduced prior to flucloxacillin use unless a test for multiple HLA alleles associated with the development of DHRs simultaneously can be implemented, as outlined by Pirmohamed et al. [148]. The clinical features of flucloxacillin-induced liver injury, and the detection of HLA-B*57:01 as a susceptibility factor suggests that predisposition relates to the development of an HLA-class I restricted drug-specific CD8+ T cell response. Wuillemin et al. [149] have demonstrated that T cells infiltrate the liver of susceptible patients. We isolated T cells from patients with liver injury and showed that flucloxacillin activates CD8+ T cells in an HLA-B*57:01-restricted manner [31]. In contrast to the drugs discussed above, flucloxacillin binds directly to lysine residues on protein and activates T cells from patients via a hapten mechanism [150]. Previous studies exploring β-lactam-specific T cell responses have shown that drug-modified proteins and peptides activate patient T cells confirming that the formation of a covalently bound drug protein adduct can lead to immune responses [108]. Despite this, the nature of naturally occurring, drug-modified immunogenic peptides have never been defined. The association between flucloxacillin and HLA-B*57:01 allows for the use of HLA-transfected cell lines to characterize the immunopeptidome in the presence and absence of flucloxacillin using the methods established with abacavir. If drug-modified peptides are identified, they can be used in future experiments to explore T cell immunogenicity. Using cells from HLA-B*57:01+ drug-naïve healthy

donors, it was also possible to prime naïve CD4+ and CD8+ T cells to flucloxacillin [71]. Interestingly, the response to flucloxacillin in HLA-B*57:01+ healthy donors seems to be driven by the direct reversible binding of soluble drug to the HLA molecule [151]. These data show the complexity of studying drug-specific T cell responses and highlight the importance of understanding what different models should and should not be used for.

1.4.3.4 CARBAMAZEPINE

In 2004, the second strong association between the development of a DHR and expression of a specific HLA allele (HLA-B*15:02) was described [152]. Carbamazepine is an anticonvulsant drug used in the treatment of epilepsy. Administration of carbamazepine is associated with a high prevalence of DRHs including severe cutaneous reactions such as Steven– Johnsons syndrome and toxic epidermal necrolysis [153]. These life-threatening conditions are differing severities of the same disease and present as an autoimmune attack on epithelial cells, leading to skin detachment. Both reactions are associated with a high morbidity and mortality [154,155]. HLA-B*15:02 was first described as an allele linked to carbamazepine-induced Stevens–Johnson syndrome. HLA-B*15:02 is much more commonly seen in Asian populations than Caucasian; hence Stevens–Johnson syndrome due to carbamazepine treatment is more frequent in Asian populations [156,157]. Europeans who develop CBZ-induced Stevens–Johnson syndrome do not commonly express HLA-B*15:02. Those that do are of Asian origin/ethnicity [156,157]. These data highlight an important difference between abacavir and carbamazepine DHRs. Reactions to abacavir are only seen in donors expressing HLA-B*57:01; whereas carbamazepine reactions occur in donors with and without HLA-B*15:02. This suggests that carbamazepine may interact with multiple HLA alleles to activate T cells. Early functional studies with

PBMC from patients with carbamazepine hypersensitivity confirm this. CD4+ and CD8+ T cells from hypersensitive patients who do not express HLA-B*15:02 are activated with the drug [115,134]. Furthermore, the activation of CD4+ T cells was HLA-DR and -DQ restricted indicating that the drug interacts with HLA class II molecules. CBZ does not need to bind covalently to protein to stimulate T cells. Instead, the drug interacts reversibly with HLA proteins and T cells are activated in a matter of seconds, much faster than the time frame observed in the hapten pathway where processing of protein adducts takes several hours [158,159]. This shows that antigen processing is not an absolute requirement for T cell activation; however, the data does not exclude a hapten pathway in carbamazepine hypersensitivity. Carbamazepine is converted enzymatically to multiple metabolites and at least three of these display protein reactivity [160–162]. The problem is that these metabolites cannot be generated in situ in immune assays and the metabolites are not available for functional studies or cannot be applied in a relevant form. For example, (1) the proteins carbamazepine modifies in patients and (2) the nature of the bound hapten structure have not been defined; as such the synthesis of immunologically relevant protein adducts is not possible. Interestingly, carbamazepine 10,11 epoxide, a major metabolite of carbamazepine that was believed to display no protein reactivity, has recently been shown to modify albumin in patients exposed to a therapeutic course of the drug [163]. Relatively high concentrations of carbamazepine 10,11 epoxide selectively activates certain clones from carbamazepine hypersensitive patients via a p-i pathway [134]. Thus, it is feasible that this in vitro p-i response is mimicking a hapten pathway of activation in patients where metabolite exposure concentrations are significantly lower. Similar to the implementation of abacavir genetic screening, the predictive values of the HLA-B*15:02 association was strong enough for the FDA to recommend genetic screening

of epilepsy patients before commencement of CBZ treatment for individuals of Chinese descent. Focusing specifically on the interaction of carbamazepine with HLA, Wei et al. [163] and Ko et al. [164] found that HLAB*15:02 shows binding affinity with the 5-carboxamide structure in the tricyclic ring of carbamazepine and that an arginine residue at position 62 of the HLA molecule was important for the direct binding of carbamazepine. Furthermore, an elegant series of experiments demonstrated that a highly restricted T cell receptor usage is observed in HLA-B*15:02+ patients that develop Stevens–Johnson syndrome. Patients lacking the specific receptors were found to tolerate carbamazepine even if they expressed the HLA risk allele. These data suggest that specific expression of a single HLA molecule and a T cell receptor determines susceptibility to this single form of DHR in Han Chinese patients. However, it should be noted that this restricted T cell receptor usage does not seem to be a major predisposing factor for other DHRs, including abacavir hypersensitivity [129]. Since the initial discovery of an association between HLA-B*15:02 and carbamazepine hypersensitivity in Asian populations, HLA-A*31:01 has been identified as a risk factor in patients with different forms of CBZ hypersensitivity in Caucasian patients of European decent. The absence of the allele in the population decreased risk of a serious DHRs from 5% to 3.8%, but important data showed that the presence of the allele augmented the risk by fivefold, from 5% to 26% [154]. In contrast to HLA-B*15:02, the interaction of carbamazepine (metabolites) with HLA-A*31:01 has not been studied in great detail. We have focused on a single HLA-A*31:01+ hypersensitive patient and isolated carbamazepine-responsive CD4+ and CD8+ T cells [165]. The drug-specific CD8+ T cell response was HLA-A*31:01+ restricted indicating that the drug interacts with the risk allele; however, the CD4+ T cells were activated in a HLA-DRB1*04:04 restricted manner. Ethnicity-dependent

additional risk factors have been identified within the HLA-B*75 serotype for carbamazepine hypersensitivity. These include: HLA-B*15:02 in Indian and Han Chinese populations, HLA-B*15:11 in Japanese populations, and HLA-B*15:21 found in South-East Asian populations, with members of the HLA-B75 serotype all displaying similar ability to bind carbamazepine, potentially leading to the induction of an immune-mediated adverse reaction [166–168].

1.4.3.5 ALLOPURINOL

Allopurinol is another of the drugs with a known HLA risk allele for DHRs. Allopurinol is used in the treatment of gout to decrease problematic high levels of uric acid in the blood. Treatment of gout with allopurinol alongside NSAIDS has been effective; however, serious adverse events can occur. Some of these reactions include the high morbidity and mortality illnesses Stevens–Johnson syndrome and toxic epidermal necrolysis, in addition to impaired renal function and hepatitis [169]. Being of Han Chinese origin, female, and over the age of 60 have been shown to be risk factors for development of allopurinol-mediated skin eruptions [170]. The risk allele HLA-B*58:01 has also been identified as a factor in whether or not a DHR will occur in patients [171], whilst genotyping patients and screening out patients with the risk allele for allopurinol treatment has proved successful in decreasing hypersensitivity reactions [172]. Experiments were conducted utilizing T cells from both HLA-B*58:01 positive and negative individuals with specificity for allopurinol and its metabolite oxypurinol. A synergistic relationship between both the presence of the HLA-B*58:01 allele, as well as the concentration of the drug resulted in the development of the DHR. Reactions involved activation of HLA-B*58:01 restricted CD8+ T cells by the metabolite oxypurinol. The drug metabolite-specific T cells were activated in a dose-dependent manner via a PI pathway [169].

1.4.3.6 Other HLA Associations – Extended

The proliferation of genome wide association studies has resulted in many more risk alleles being identified in recent years. Please see table 1.1 for an overview of some other purported HLA class I and II allele risk associations.

Table 1.1 - HLA alleles associated with DHRs. Table in part adapted from Ghattaoraya et al., Allele frequency.net web archive.

Class I associations		Class II associations	
Drug	HLA allele	Drug	HLA allele
Abacavir	*B*57:01*	Aspirin	*DPB1*03:01*
			*DQB1*06:09*
Allopurinol	*A*33:03*	Azathioprine	*DQB1*
	*B*58:01*		*DRB1*
Carbamazepine	*A*31:01*	Clozapine	*DQA1*03:01*
	*B*15:02*		*DQB1*03:01*
	*B*51:01*		*DQB1*05:02*
Clozapine	*B*38*	Co-amoxiclav	*DQA1*01:02*
			*DQB1*06:02*
			*DRB1*15:01*
Co-amoxiclav	*A*02:01*	Efavirenz	*DRB1*01*
Dapsone	*B*13:01*	Lapatinib	*DQA2*02:01*
			*DQB1*02:02*
			*DRB1*07:01*
Flucloxacillin	*B*57:01*	Lumiracoxib	*DQA*01:02*
			*DQB1*06:02*
			*DRB1*15:01*
Lamotrigine	*B*13:02*	Nevirapine	*DRB1*01:01*
	*B*15:02*		*DRB1*02:01*
Methazolamide	*B*59:01*	Ximelagatran	*DQA1*02*
			*DRB1*07*
Nevirapine	*B*35:05*		
Oxcarbazepine	*B*15:02*		
Phenytoin	*B*15:02*		
Sulfamethoxazole	*B*38*		
Ticlopidine	*A*33:03*		
	*B*43:03*		

1.5 IMMUNE REGULATION

The drug-MHC-TCR interaction is pivotal for the initiation of an immune response; the formation of this immune synapse is a determinant of immune activation. Following the successful formation of an immune synapse, TCR clustering and T cell activation occurs through the induction of a signalling cascade triggered by TCR phosphorylation by LCK and subsequent recruitment of ZAP70 through the LAT signalling pathway. MEK/ERK signalling alongside the other signalling pathways lead to CD69 upregulation which is a key factor of T cell activation [173]. Ca2+ influx also leads to cellular production of interleukin-2, which is a key cytokine involved in T cell activation and survival; this is part of signal 3 which will be discussed later.

However, the formation of an immune synapse alone (signal 1) is not sufficient to invoke an immune response. Indeed, there are a multitude of other signals that have a determining factor on lymphocyte activation, and the observable outcomes *in vivo*. These are termed signal 2 and 3 and can be placed under the umbrella of immune regulation alongside cellular immune regulation and transcriptional regulation. Some examples of immune regulation that are relevant to this thesis will be discussed below.

1.5.1 SIGNAL 2

1.5.1.1 CO-SIGNALLING

Co-signalling is formed of a plethora of both co-stimulatory and co-inhibitory signals; some of which are expressed in isolation, and some that are overlapping and directly competitive. These pathways play a major role in the modulation and outcome of T cell activation, as signal 2 (especially co-signalling pathways) have a direct effect on whether or not signal 1 occurs and T cell activation threshold is achieved leading to lymphocyte activation and proliferation. If signal 1 occurs in the absence of signal 2, then tolerance is observed. Likewise, if signal 1 is observed alongside a co-inhibitory

signal 2, then tolerance and cellular anergy occur [98]. However, if signal 1 accompanied by a co-stimulatory signal 2, then the T cell activation threshold can be achieved, triggering T cell activation after the processes (described in the previous section) have come to pass. Indeed, it has been said that *in vivo* there is a balancing act between tolerance and anergy, that is influenced by a signal 1: signal 2 axis.

The co-signalling pathways have become an important therapeutic target over recent years; as scientists have discovered that these pathways may hold the key to the modulation of the immune system. Thus, one can develop novel therapies for diseases with an immune aetiology; including auto immune disorders and cancers [174,175]. Indeed, it has been regularly reported that co-signalling plays a vital role in immune homeostasis, and mutations in co-signalling members such as PD-1 and CTLA-4 has led to Treg dysfunction and autoimmune disease in both mice and humans [176]. Cancerous tumour cells have developed mechanisms to shield themselves from host immune system by relying on the effectiveness of the hosts co-signalling pathways and self-tolerance mechanisms; including PD-1 and CTLA4. It has also been reported that tumour cells can increase PD-L2 expression levels on inhabited macrophages to evade destruction by host immune cells [177]. Thus, novel immunotherapies based on the blockade of co-signalling receptors may pave the way for the destruction of tumerous cancer cells by host immune systems [174,178].

Despite this breakthrough in cancer immunotherapy, progress has been impaired by some devastating adverse events observed upon treatment with co-inhibitory signalling blockade *in vivo* [179]; perhaps further indication of the influence these pathways have on the initiation of an immune challenge.

There are many different receptor-ligand interactions that constitute the full range of co-signalling pathways. Some of the co-stimulatory members include OX40, CD137,

CD28 and ICOS [180,181]; whilst some of the co-inhibitory signalling pathways include PD-1, CTLA-4 and TIM-3 [137,182–184]. Below will be an explanation of the co-signalling pathways pertaining to research in this thesis (CD28 co-signalling family, including: PD-1, CTLA-4, CD28) as the direct effects of these pathways on drug immunogenicity have been investigated in recent years 3 [137,182], but their full effects upon DHR need to be further investigated.

PD-1 is constitutively expressed on a wide range of immunological cell types such as T cells, NKT cells, NK cells and monocytes, but also throughout the body at other sites with a heavy immune presence such as the thymus [183]. In order to exert its co-inhibitory effects on the immune system, PD-1 must interact with one of its two known ligands; PD-L1 or PD-L2. PD-L1 is expressed on a range of immune cells and is induced by IFN signalling, whereas PD-L2 is expressed in a more focused manner on APCs such as DCs, activated macrophages or mast cells and is induced by IFN-γ, GM-CSF, and IL-4 [185]. The interaction of PD-1 with either of its ligands has been shown to suppress antigen-specific T cell responses [186]; whilst other studies have highlighted that abrogation of PD-1/PD-L1 binding can restore or increase antigen-specific T cell response [137,182,187]. This interaction between PD-1 and either of its ligands is known to perturb the T cell activation cascade as described in section 1.5; as recruitment of SHP proteins leads to dephosphorylation of ZAP70, affecting TCR clustering, preventing further downstream signalling such as AKT and MEK/ERK signalling leading to T cell activation [188]. Fife et al., also explained eloquently that PD-1 causes immune suppression via the prevention of signal 1 establishment, as the blockade of PD-1 causes increased contact time between T cells and DCs [188]. These immune dampening effects of PD-1 is also linked to decreased IL-2 expression, which is a key cytokine involved in T cell activation and survival [189]. As previously mentioned, PD-1 is an

effective immunosuppressive tool that is utilised by cancerous cells to evade host immunity and survival of tumour cells. This thesis will further investigate its purported role in DHR; demonstrated by its pivotal involvement in T cell response.

CTLA-4 is another major immune checkpoint that is involved in cancer cells. CTLA-4 deficient mice have been shown to die within weeks of birth; often succumbing to lymphoproliferative disorders [190] which highlights CTLA-4 involvement in immune homeostasis. Like PD-1, CTLA-4 interacts with two ligands, in this case CD80 and CD86 to exert its effects. Unlike PD-1, CTLA-4 is in direct competition with the co-stimulatory CD28 for binding to the CD80/CD86 ligands to determine the outcome of signal 2 in these instances. CD80 has been described as predominantly co-stimulatory, whilst CD86 is mainly co-inhibitory [191]. Despite its lower expression than CD28, CTLA-4 has a higher affinity for the CD80/86 ligands and binds to them more readily. If CTLA-4 competitively inhibits CD28 binding, then co-inhibitory effects and tolerance are observed [58]; whereas CD28 victory would determine co-stimulatory effects, lowering T cell activation threshold, and immune activation. CTLA-4 can also regulate immune responses through its effects on Treg-APC binding competition; as Treg binding to APCs inhibit the availability for the formation of a co-stimulatory immune synapse, in addition to Treg immunosuppressive effects [58,59]. This action is mediated through CTLA-4 (which is constitutively expressed on Tregs) and CD28 competition, as CD28 superagonist therapies such as TGN1412 were originally devised to modulate these interactions; reactivating Tregs in T cell deficient diseases such as rheumatoid arthritis and B cell leukaemia. The vital role of CD28/CTLA-4 interplay in co-signalling and determination of immune outcomes was highlighted by the failure of TGN1412; as overactivation of the CD28 receptor led to overactivation of the immune system, concluding with cytokine storm and organ failure in

participants of the clinical trial [192,193]. CD28 involvement in immune stimulation was also observed in the early nineties, as anti-CD28 antibodies were used to induce T cell anergy [181].

1.5.1.2 DANGER SIGNALLING

Whilst direct covalent binding of a drug to target cells in an organ is thought to lead to potential adverse events (iDILI from flucloxacillin covalent binding to hepatocytes), this does not always tell the whole story. Some studies have shown that the level of covalent binding of flucloxacillin detected is not directly related to incidences of iDILI [194]. As signal 2 is implicated in whether or not signal 1 can be established, there must be some other exterior influences in this instance.

Matzinger, in 1994 developed the first hypothesis explaining that the immune system can be activated in response to certain signals termed 'danger signals' which were often as result of cellular stress and damage. But the lack of these signals can lead to immune tolerance and anergy [195]. These danger signals can be subdivided into two categories: DAMPS and PAMPS. DAMPS can be intracellular components, released during necrotic episodes in response to drug or reactive drug metabolite toxicity and interact with TLRs on DCs; upregulating signal 2 to induce immune response. HMGB1 is an established biomarker for DILI and is deemed a DAMP as it is released by necrotic cells, but not apoptotic cells; determining the cellular damage response [77,196]. Similarly, studies within the group on chemical sensitizers and chemically reactive metabolites arising from experimental drugs such as SMX-NO have been shown to activate DCs through TLR modulation, this would then lead to co-stimulatory activation on DCs and T cell activation [80]. Interestingly, small molecules and CRMs such as SMX-NO can be danger signals themselves as described immediately above but can also influence further downstream danger signalling such

as the release of DAMPs through their cytotoxic effects on cells [110,197]. PAMPS, whilst not as directly relevant to this thesis as DAMPS, still play an important role in T cell activation. Lipopolysaccharide (LPS) in gram negative bacteria is a known PAMP and can be used in maturation of monocyte derived DCs. Other pathogens such as HIV are thought to be a factor in why HIV treatment so often leads to adverse events as the innate immune system interacts with the extracellular HIV pathogen before the addition of a chemical antigen leads to full immune activation [198].

1.5.2 SIGNAL 3 – CYTOKINES

Cytokines are small, low molecular weight signalling molecules that are released from a range of immune cells (including T cells, B cells, DCs, NK cells and macrophages) and locate to their specific receptors on target immune cells and tissues [199,200]. They are critical for the correct function of the host immune system and are the major communicative pathway between leukocytes and their respective immunological partners. They enable the communication between immune cells, the skewing of phenotypes of T cells that is described by T cell plasticity (T_h1/T_h2), differentiation of immune cells (such as monocytes to DCs), the maturation of immune cells such as DCs which help them fulfil their function, and are also directly involved in cellular processes which enable immune cells to carry out their function directly; such as release of FasL and perforin from CD8+ cytotoxic T cells. The influence of cytokines on a multitude of different signalling pathways mean their function and interactions are endlessly complicated; they are referred to as the cytokine milieu. Therefore, they have a large influence on whether or not an immune mediated event occurs, as well as the strength of said response in its outcome. The above is also described in section 1.3.

Examples of cytokines used experimentally in this thesis during cell culture are: GM-CSF and Il-4. These were used during the culture of CD14+ monocytes isolated from

healthy volunteers and their subsequent differentiation to DCs [201]. Pro-inflammatory cytokines such as TNF-α can be used to promote cellular maturation, and so TNF-α was used during DC culture to promote maturation and improve antigen presenting capability in naïve T cell priming cultures [202], alongside LPS (classified as a PAMP). As IL-2 is one of the most important cytokines related to T cell function (activation and proliferation) [203], we have used it for the re-stimulation and culture of antigen specific T cell clones in chapter 6. Like many other cytokines, once contact has been made between IL-2 and its receptor, a downstream JAK/STAT mediated signalling cascade is activated to elicit its effects; the activation and survival of T cells, especially Tregs are directly influenced here [203–205]. Cytokines can also be detected by ELISA, ELISpot, or intracellular staining flow cytometry to determine the effects of cells in response to certain treatment conditions. Pro-inflammatory cytokines include TNF-α, IFN-γ, IL-1 and IL6 – whilst anti-inflammatory cytokines include IL-10 and IL-12. Cytokines such as IL-2, IL-4, IL-7, IL-9, IL-13 and GM-CSF interact almost exclusively with the adaptive immune system. The above are also described in section 1.3.

Cytokine production can be effectively used as part of immunomodulatory pathways and immune regulation. However, instances such as treatment with CD28 superagonist TGN1412 - leading to uncontrolled cytokine secretion (cytokine storm) - can have severe consequences to the treated party [192,193]. This outlines the importance of cytokines for the proper function of the immune system in a healthy individual; any change to this can influence adverse events and potentially form part of the pathogenesis of DHR.

1.5.3 CELLULAR REGULATION – TREGS

In addition to signal 2 and 3 described above, immune regulation can also take place at the cellular level. Tregs have an extremely important immunoregulatory function, and can be involved in the modulation of immune responses in either a immunosuppressive state, or a pro-inflammatory state. Furthermore, they become increasingly relevant within the context of this thesis through their immunoregulatory effects, as they directly interact with other members of the immunoregulatory family; specifically the signal 2 CD28 family [59]. More detail on Tregs can be found in section 1.3.2.

1.5.4 MICRO RNA

Post-transcriptional regulation of the immune system has also become an increasingly important focus of research in recent years. The discovery of miRNAs as biomarkers of biological processes have been used to predict adverse events - circulating miR-122 in serum for the prediction of DILI [206,207] has provided a less-invasive method of rapid diagnosis of DILI; however, a widespread reliable diagnostic test has yet to be established. There has now been an explosion of research into miRNAs as regulators of immunological processes; including the pathogenesis of DHRs.

MiRNAs are single stranded 22 nucleotide RNAs that regulate gene expression by binding to the 3′ untranslated region (UTR) of their target, promoting translational repression or the direct degradation of the mRNA. Upregulation of a specific miRNA promotes higher inhibition of the target gene which manifest in phenotypic and physiological changes in the cell [208]. MiRNAs are involved in regulating the expression of 30-50% of genes; and so are involved in many key physiological and fundamental cellular processes [209]. They can elicit regulation that alters the concentration of key protein components of cascading signalling pathways of immune

activation at the post-transcriptional level, and importantly, studies have shown that genetic ablation of miRNA machinery has led to auto-immunity [210]. MiRNAs are becoming notorious as key regulators of important biological processes; such as proliferation, differentiation and development, as well as crucial regulators of both innate and adaptive immune responses [211]. This occurs through a purported role in the induction, maintenance and function of Tregs, and regulating the differentiation of APCs, DCs and macrophages through TLRs [212].

Over a hundred miRNAs have been shown to be expressed by immune cells and have an impact in basic immunological process [213]. Despite the wide variety of miRNAs showing an altered expression, only a few have been studied in detail. These include miR-9, miR-17 ≈ 19 cluster, miR-21, miR-146a, miR-155, miR-181a, and miR-214. For most of these, their target genes have been validated and their function has been evaluated during T cell activation by functional assays [214–220]. MiRNAs have also been implicated in the regulation, maintenance and function of Tregs, in addition to involvement in the co-signalling pathway; PD-L1 specifically. MiRNA binding to PD-L1 mRNA on APCs can lead to degradation and ultimately suppression of the ligand and therefore overall suppression of the PD-1/PD-L1 axis, resulting in increased immune activation. MiR-513, miR-570, miR-34a, and miR-200 reportedly display inverse correlation to PD-L1 expression [221]. MiRNAs have also been directly implicated in DHR through targeting and post-transcriptional regulation of the granulysin gene [222].

1.6 THESIS AIMS

There is a desperate need for reliable, appropriate *in vitro* T cell assays for the prediction and diagnosis of DHR. Whilst the standard DC-naïve T cell priming assay can enable us to determine the likelihood of a drug to prime healthy volunteer naïve T

cells, there are some limitations with the assay that need to be resolved before the assay is ready for widespread pre-clinical use which have been discussed previously. Additionally, the LTT is the current gold standard for *in vitro* DHR diagnosis, but as previously mentioned also has some issues; such as the time taken to complete the assay.

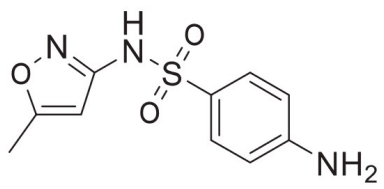
The aim of the thesis is to evaluate and then improve upon the current *in vitro* methods of predicting and diagnosing DHR. This leads to the development of two new assays to form part of a predictive screening system for the *in vitro* prediction of DHR. These assays must be able to analyse naïve T cell priming in multiple donors on a single plate and take into account the naïve T cell precursor frequency required for activation. Furthermore, we aim to investigate the role of immune regulation (at the cellular and transcriptional level) on the priming of naïve T cells to a drug, with a view to implementing our findings to improve our new *in vitro* assays. Finally, we aim to investigate whether the LTT is fir for purpose, with an aim to improve the *in vitro* diagnosis of DHR.

1.7 EXPERIMENTAL COMPOUNDS IN DRUG ALLERGY

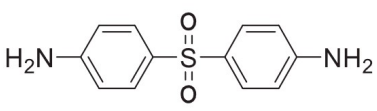
Below is a summary of the drugs used in experiments throughout this thesis.

1.7.1 CHEMICALLY REACTIVE METABOLITE NITROSO-FORMING DRUGS

Sulfamethoxazole



Dapsone



Sulfamethoxazole (SMX) is a sulfonamide bacteriostatic antibiotic, often used in combination with trimethoprim for the treatment of opportunistic infection in both

HIV patients and recurring infections in CF patients. Whereas dapsone (DDS) is a diaminodiphenyl sulfone used in combination therapy with rifampicin and clofazimine for the treatment of leprosy. Despite their different classification, both have NH_2 groups attached to a benzene ring as can be seen above. SMX has been implicated in severe DHR reactions in up to 3-8% of the population, with DHR prevalence known to increase in HIV patients with an active infection [133,223]. In SMX hypersensitivity both the hapten and p-i mechanisms of activation have been implicated. However, antigen specific T cells of sensitised patients were deemed reactive to both the parent drug SMX, and the reactive metabolite SMX-NO which is formed by liver metabolism via an SMX-hydroxylamine intermediate, before autooxidation to the reactive nitroso-metabolite [111,223]. Further research within the group over the years has shown SMX-NO to be an excellent test-compound; acting as a 'model hapten'. SMX-NO is more reactive than the parent drug SMX, responding in 9/10 tolerant individuals opposed to 3/10 [111]. SMX-NO has also been found to have the propensity to activate DCs through in a DAMPS mediated manner, and can activate co-signalling CD40 on APCs to induce immune responses [80]. SMX-NO has been proven to consistently prime healthy donor naïve T cells in T cell priming assays making it an excellent candidate for use in this thesis [71,135,137,182]. Dapsone, like SMX, also forms a nitroso reactive metabolite (DDS-NO), and has been implicated in DHR [224]. DDS-NO involvement in DHR is less established than SMX-NO, but recent studies in the group have aimed to rectify this with research showing a HLA-B*13:01 restricted CD8+ T cell responses to DDS-NO, but not DDS in healthy donors [144,225]. For this reason, DDS-NO was also used experimentally in this thesis, albeit to a lesser extent than SMX-NO.

1.7.2 BETA-LACTAM ANTIBIOTICS

Beta-lactams are a class of antibiotics that are identified through the presence of the beta-lactam ring contained within their molecular structures. Beta-lactams include penicillins, cephalosporins, carbapenems and monobactams which are used in the treatment of bacterial infection. Despite their widespread use as a treatment strategy for bacterial infection, beta-lactams are also renowned for their contribution to adverse events and DHRs. Indeed, they are the most common cause of immune mediated ADRs. Both beta-lactams used experimentally within this thesis have been implicated in such events; piperacillin has been found to cause both immediate adverse reactions, and also delayed type IV hypersensitivity skin reactions such as macropopular exanthema and uticaria [226,227]. Flucloxacillin on the other hand has been implicated in drug induced liver injury [228]. Beta-lactam's known pathomechanisms takes place through covalent binding, modified drug-protein adducts have been investigated throughout the years as a potential cause of DHR as modified drug-protein adducts have been found to activate T cells directly [229]. The opening of the beta-lactam ring has been determined to be the major antigenic pathway for this class of drugs [230], which under normal working conditions specifically target certain lysine residues on HSA to enable favourable binding conformations. At higher concentrations, or after long-exposure times beta-lactams can target multiple residues as opposed to the favoured lysine [150,230]. Work within the group have functionally characterised piperacillin specific drug reactive T cells *in vitro* to understand their involvement in skin related DHR. Expansion of circulating and skin-resident T_h22 cells that secrete IL-22 but not IL-17 have been implicated in such reactions. Whilst skin-homing of T cells occurs due to the expression of chemokines CCL17 and CCL27 [50]. Flucloxacillin-specific T cell clones were found to secrete cytolytic granzyme B and

FasL and perforin, as well as IFN-γ. Flucloxacillin activation was HLA-B*57:01 restricted and processing dependent, with covalent binding to specific lysine residues on albumin correlating with T cell activation [31]. For these reasons, the beta-lactams piperacillin and flucloxacillin were deemed to be worthy of further investigation and used as test compounds during the development of our screening assays for DHR.

1.7.3 CONTACT SENSITIZERS

Bandrowski's base (BB) was also used as a test compound in the development of T cell assays. BB is formed as an oxidised product of p-phenylenediamine (PPD) which is used throughout the cosmetics industry in hair and tattoo dyes, meaning that many people will have become sensitised [104]. PPD has been implicated in both immediate and non-immediate reactions, with a purported patho-mechanism of PPD-keratinocyte binding and interaction with Langerhans cells, whilst BB has been reported to be ten times more potent than PPD as a contact sensitizer [231]. Due to its severe and potent reactivity with the skin and its ability to activate T cells, it was decided to be an important test compound to test the ability of *in vitro* naïve T cell priming. Indeed it has the propensity to prime naïve T cells in our standard naïve T cell priming assays in a strong and reproducible manner [104,232]. However, data has shown that PPD only activates T cells in allergic patients, not healthy volunteers; whilst BB can activate T cells in both cohorts. Hence its inclusion in our experiments on healthy volunteer cells in this thesis [233].

2 MATERIALS AND METHODS

2.1 REAGENTS AND MATERIALS

The reagents and materials used throughout this thesis and where they were obtained from can be found below:

- Lymphoprep was purchased from Axis-Shield (Dundee, UK).
- Recombinant IL-2, GM-CSF, IL-4 and TNF-α was from Peprotech (London, UK).
- Propan-2-ol was purchased from Fischer Scientific (Loughborough, UK).
- Di-methyl sulfoxide was from Sigma-Aldrich (Dorset, UK).
- Fetal bovine serum was from Invitrogen (Paisley, UK).
- Human serum albumin was from Innovative Research (Michigan, USA).
- [3H]-methyl tritiated thymidine was from Moravek (California, United States).
- Sulfamethoxazole-nitroso was from Dalton Pharma Solutions (Toronto, Canada).
- Piperacillin was from Wrockhardt Limited (Wrexham, UK).
- Tetanus toxoid was from Statens Serum Institut (Copenhagen, Denmark).
- Flow cytometry fluorescent antibodies were from R&D Systems (Minnesota, USA) or BD Bioscience (Oxford, UK).
- All ELISpot kits were from Mabtech (Stockholm, Sweden).
- ELISpot plates were from Millipore (Watford, UK).
- Melt-on wax scintillator sheets, glass fibre filter mats and plastic sample wallets were purchased from Perkin-Elmer Life Sciences (Massachusetts, USA).
- Magnetic bead separation kits were from Miltenyi Biotech (Surrey, UK).

- Cell culture plates, flasks, dishes and other assorted plastic-ware were purchased from Thermo Scientific (Hemel-Hempstead, UK).
- TRI-reagent for RNA extraction was from Sigma-Aldrich (Dorset, UK).
- Universal cDNA synthesis kit was from Exiqon (Copenhagen, Denmark).
- SYBR Green master mix and primer kits were from Exiqon (Copenhagen, Denmark).
- All other regents and consumables related to RNA work, PCR, or qPCR were obtained from Qiagen (Manchester, UK).
- Unless otherwise stated, any other reagents or supplies were obtained from Sigma-Aldrich (Dorset, UK).

2.2 BUFFERS AND CELL CULTURE MEDIUMS

Standard lymphocyte cell culture medium contained:

- RPMI
- 10% HSA
- Penicillin (100U/ml)
- Streptomycin (0.1mg/ml)
- Transferrin (25µg/ml)
- L-glutamine (2mM)
- HEPES buffer (25mM)

Cell freeze mix contained 1:1 ratio of the above T cell culture medium, supplemented with:

- 80% FBS
- 20% DMSO

FACS Buffer (Flow cytometry medium) contained:

- Hanks balanced salt solution (HBSS)
- 10% foetal bovine serum
- 0.03% sodium azide (NaN3)

MACS buffer (Magnetic cell separation medium) was made to 10x stock, (subsequently diluted to 1x with HBSS) using:

- 2.5g BSA
- 2mL EDTA (500mM)
- 48mL HBSS

2.3 INSTRUMENTATION

The following instruments were used experimentally for the generation of data in this thesis:

- Cell harvester (assays using thymidine incorporation) was from TomTec (USA).
- Micro Beta Trilux Counter for counting harvested T cell assays was from Perkin Elmer (Cambridge, UK).
- ELISpot plates were read using AID ELISpot Reader from Cadama Medical (Stourbridge, UK).
- MRX plate reader from Dynex (Lincoln, UK) was used to quantify ELISA assays.
- BD FACS Canto II from BD Biosciences (Oxford, UK) was used in all flow cytometry experiments.
- Flowing 2 was used for flow cytometry analysis (Turku University, Finland).

- All instruments related to magnetic cell subset separation were obtained from Miltenyi biotech (Surrey,UK).
- Nanodrop 2000 from Thermo Scientific (USA) was used to quantify RNA.
- Quantitative PCR was performed using a ViiA 7 Real-Time PCR System (Thermo Scientific, USA)
- Qiacube from Qiagen (Manchester, UK) was used to fill the PCR plates with samples.

2.4 BLOOD SAMPLES

Approvals for studies were obtained from the Liverpool local Research Ethics Committee, informed written consent was acquired from all participating blood donors (including healthy volunteers). Hypersensitive paediatric patient samples (Chapter 5) were obtained from either: Regional Cystic Fibrosis Unit at St. James's University Hospital, Leeds, UK, or the Royal Manchester Children's Hospital, Manchester, UK.

2.5 EXPERIMENTAL PROCEDURE

2.5.1 PBMC ISOLATION

Peripheral blood mononuclear cells were obtained from either healthy donor or patient blood samples. Blood was taken in accordance with ratified studies and ethics committees. Blood arrived in heparinised blood vials before being layered with syringe and quill in a 1:1 ratio into a 50mL falcon tube with lymphoprep. Tubes were centrifuged (25 minutes, 400g, room temperature, moderate acceleration, brake deactivated) which generated a buffy coat. Buffy coat containing PBMC was carefully extracted using a Pasteur pipette before being washed twice in 50mL HBSS (15-minute centrifugation, brake on full, room temperature). Viable cells were then counted using trypan blue exclusion method in 1:1 ratio. Cells were frozen at 10,000,000/mL at -80 degrees Celsius overnight before being transferred to either -

150 degrees Celsius freezer, or liquid nitrogen for longer term storage. Cells could then be carefully thawed, spun down and re-counted before being used in any of the following experiments.

2.5.2 Separation of Cell Subsets

PBMC were re-suspended in MACS buffer at 800uL/1x10^8 cells before being incubated in the dark at 4 degrees Celsius for 15 minutes with CD14 microbeads (200uL/1x10^8 cells). Cells were washed in MACS buffer (centrifuged at 4 degrees Celsius, 10 minutes, 400g) before being re-suspended in MACS buffer (500uL/1x10^8 cells) and passed through an LS column mounted to magnetic stand. $CD14^-$ cells passed through the column, whilst $CD14^+$ cells were eluted from the column by plunging MACS buffer through the column. Cells were then counted, $CD14^-$ cells were then used for subsequent cellular isolations.

All subsequent cell separations were performed under the same conditions, the only difference being the type of microbeads used, and the amount of microbeads added. They were used as follows: Pan-T biotinylated antibody cocktail (100µl per 1x10^8 cells) was used to isolate *all* $CD3^+$ cells, anti-biotin microbeads (200µl per 1x10^8 cells) were used in conjunction with the antibody cocktail for this cell isolation. Flow through cells were $CD3^+$ and were used for further cell isolations. $CD3^-$ cells were frozen down and contained B cells which could be used for EBV transformed B cell generation.

CD25 and CD45ro microbeads were used to positively select out Tregs and memory T cells, leaving the negatively selected cells ($CD45ro^-$) which were naïve T cells. All cells were then frozen down using previously described freeze mix and stored for future use or could immediately be incorporated into experimental assays.

2.5.3 LYMPHOCYTE TRANSFORMATION TEST

LTT was used to detect antigen-specific T memory cell responses to test compounds as previously described by Pichler *et al.* [116]. PBMC were assayed into a 96-well U-bottomed cell culture plate in triplicate at $1.5x10^5$/well with a range of drugs in different concentrations (100uL each to a final volume of 200uL/well). Cell culture medium alone was used as a negative control, whilst tetanus toxoid (0.5µg/ ml, 100µl) was used as a positive control as most of the adult population will have previously been sensitized to this. For the paediatric LTT assays, PHA was used as the positive control as children may not have been sensitized to tetanus toxoid at that point. Plates were then incubated for 6 days (37°C, 5% CO2). Sixteen hours prior to the end of the experiment, ^{3}[H] thymidine was incorporated into the assay (0.5µl/well) before being harvested onto a fibreglass filtermat and sealed by wax scintillation. Sealed plates were then counted on a beta-counter and CPM (count per minute) values were obtained.

2.5.4 NAÏVE T CELL PRIMING ASSAY

The 'original' naïve T cell priming assay was utilised as described previously by researchers in the Professor Dean Naisbitt research lab [71].

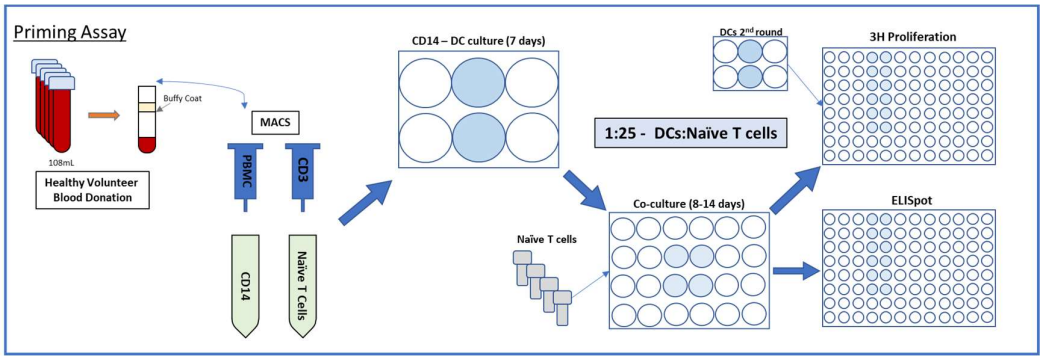


Figure 2.1 – Standard T cell priming assay. Described below.

This assay was used to prime naïve T cells from healthy volunteers to drugs and compounds. It is the original assay developed by our lab that was the start point for the further development of the screening assay systems in Chapter 3. It makes use of naïve T cells, mature DCs (as APCs), and a drug/compound of choice.

CD14+ monocytes were cultured in the presence of GM-CSF (800U/mL) and IL-4 (50µg/mL) for 6 days in a 6-well plate; fed every other day with cell culture medium supplemented with GM-CSF and IL-4. TNF-α (50µg/mL, 3µL/mL) and LPS (1mg/mL, 6µL/mL) were added on day 6 for 16 hours to induce maturation of the monocyte derived DCs to moDCs.

moDCs were scraped from 6 well plate and plated in a 24-well cell culture plate ($2x10^4$/well) with naïve T cells ($2.5x10^6$/well) along with the optimal concentration of drug/compound to prime the naïve T cells. Plates were incubated at 37˚C, 5% CO_2.

After 7 days T cells were removed and washed in HBSS before being plated in triplicate in a 96-well U-bottomed plate ($1x10^5$/well) with a fresh round of moDCs $1x10^4$/well along with either cell culture medium (negative control), a range of test drug/compound concentrations, or PHA (5µg/mL). Plates were incubated for 48 hours, at which point tritiated ^{3}[H] thymidine was added to all wells for 16 hours. Plates were then harvested, sealed and counted as previously described.

2.5.5 T CELL MULTI-WELL ASSAY (T-MWA) & T CELL MULTI-DONOR ASSAY (T-MDA)

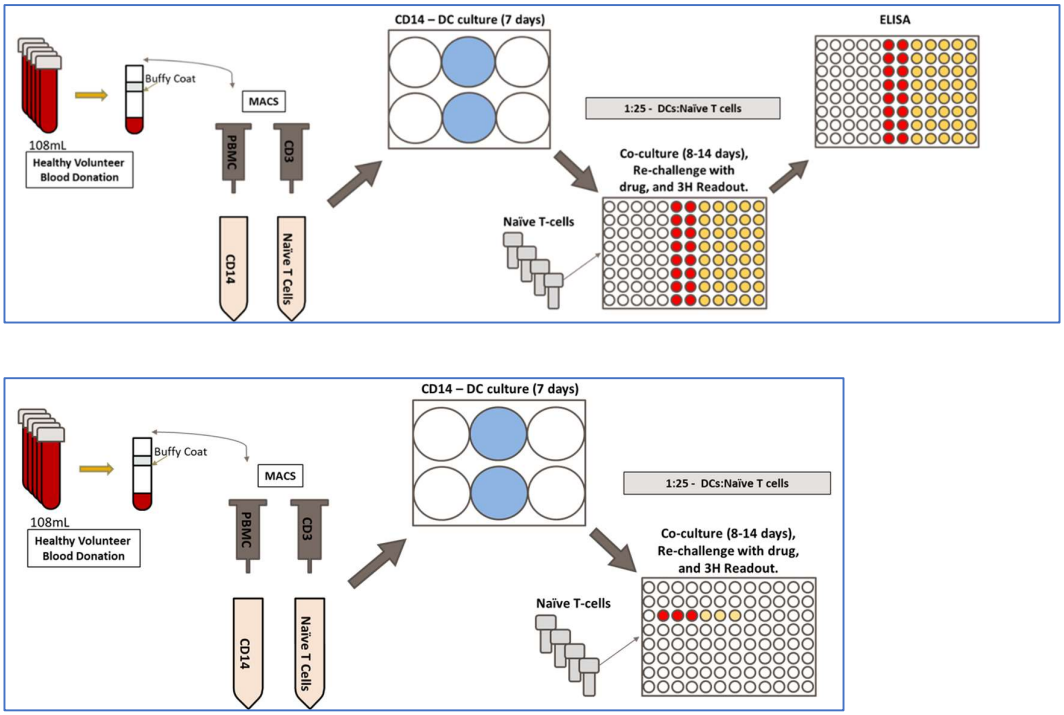


Figure 2.2 – T-MWA & T-MDA assays. Described below.

The T-MWA and T-MWA assays were developed to be a higher throughput version of the standard naïve T cell priming assay. Thus, DCs and naïve T cells were plated in a 96-well plate from the start instead of the 24-well plate used previously. The whole experiment would take place in the same plate, and as such, no second round of moDCs are needed. Due to the numbers of replicates being plated in a single assay, it would be easier to look at the priming of naïve T cells to an antigen in more detail in the T-MWA. Additionally, the precursor frequency of activation of T cells would be taken into consideration by this assay due to the numbers being plated up and primed simultaneously.

In the T-MDA it is easy to screen multiple donors simultaneously, in order to rapidly determine the likelihood of response across multiple, phenotypically different, naïve

T cell donations. This is because the cultures are set up with three wells of negative control (cell culture medium only), and three wells of drug treatment, enabling 16 donors per plate to be assayed.

Another benefit of the new screening assay system is that as a 96-well plate is used throughout, fewer DCs are needed to fulfil the experiment, and fewer naïve T cells are required per well, enabling the different plating configurations observed in the T-MWA and T-MDA.

CD14+ monocytes were cultured to moDCs as previously described before being plated directly in a 96-well U-bottomed plate ($8x10^3$/well) in co-culture with naïve T cells ($1x10^5$/well) and drug/compound at optimal concentration. Co-signalling blockers (PD-L1 and CTLA-4) could also be added at this point. In the T-MWA cells were cultured as 16 wells of negative control (media only), and 40 wells of test drug/compound treatment. In the T-MDA, multiple donors could be plated in a single 96-well plate. For each donor, 3 wells of negative control were plated, and 3 wells of drug/compound treatment were assayed. Plates were incubated for 14 days at 37˚C, 5% CO_2. On day 14, plates were carefully washed three times in cell culture medium by centrifugation (2200rpm 4 minutes). Cells were then re-suspended in cell culture medium and re-challenged with drug for 48 hours, after which point ^{3}[H] thymidine was added for 16 hours. In the T-MWA assays, supernatant can be removed at this point for analysis by ELISA assay as described in section 2.5.10. Plates were harvested, sealed, and counted as previously described. T-MWA data can be displayed as scatter plots of CPM values (with Mann-Whitney statistical test applied), or SI values can be calculated as previously described. SI values were categorised as negative, weak, good, or strong as previously described by Faulkner *et al.,* [71,135]. T-MDA data can be displayed as SI values, or by bar graph of CPM averages.

2.5.6 FLOW CYTOMETRY

Flow cytometry was used to analyse expression of cell-surface and intracellular proteins. Cells were washed before being re-suspended in FACS buffer and 5uL of fluorescently conjugated antibodies for 25 minutes in the dark. After this time, cells were washed and re-suspended in either 200uL FACS buffer for immediate analysis, or 200uL 4% para-formaldehyde for analysis at a later point. Cells were then analysed using a BD Bioscience FACS Canto II, FCS filed were analysed with Flowing 2 software. For intracellular staining, cells were fixed and permeabilised before antibody incubation using a cell fixation and permeabilization kit from Thermo Scientific.

2.5.7 T CELL CLONING

T cell cloning is a method used to be able to mechanistically analyse drug-specific T cells. The T cells produced using this method are specific to the antigen used and are expanded from a single T cell clone and originate from a single precursor cell. This is opposed to a population of drug-specific T cells that may be phenotypically different.

PBMCs were cultured in a 48-well plate ($1x10^6$ cells/well) with the drug of interest to a final volume of 1mL/well for 14 days to generate bulks. IL-2 was used to stimulate T cell activation and proliferation on days 6 and 9.

On day 14 the bulks were harvested and plated into 96-well U bottomed plate as serial dilutions; cell concentration of either 0.3, 1, or 3 cells per well. Cells were then re-stimulated with allogeneic irradiated PBMC ($5x10^4$/well), PHA (2µg/mL) and IL-2 (75U/mL). Cells were fed every 2 days with standard cell culture medium with IL-2 (75U/mL). Wells that were proliferating were picked and placed into a new 96-well plate before being re-stimulated as previously described. Picked cells were split as required and were continued to be fed every other day.

The T cell clones were tested for antigen specificity using a standard proliferation assay where an SI>2 was deemed positive. SI was calculated as CPM of drug treated wells divided by the CPM of the negative control wells (media). Alternatively, the ELISpot test was used to determine cytokine secretion of the antigen specific T cell clones.

2.5.8 PROLIFERATION ASSAY

$5x10^4$ T cell clones per well were plated in a 96-well U-bottomed cell culture plate with $1x10^4$ irradiated autologous EBV transformed B cells to a volume of 100uL. Wells were made to a final volume of 200uL with either cell culture medium (negative control), drug at the optimum concentration, or PHA as a positive control (5ug/mL). Plates were incubated for 3 days; 16-hours prior to the end of the experiment 0.5uCi/well tritiated ^{3}[H] thymidine was added to all wells. Plates were then harvested, sealed and counted as previously described.

2.5.9 ELISPOT

ELIspot, like the ELISA, can measure cytokine secretion. The main difference being that the ELISpot measures cytokine secretion from T cells, rather than using the supernatant (as in the ELISA). 96-well ELISpot (high protein binding plates from Millipore, USA) were pre-wet with 70% ethanol and then washed with distilled water. Wells of the plate were then coated with 100uL of the appropriate antibody (diluted as per manufacturer instruction) and incubated at 4˚C overnight. Plates were washed 5 times with HBSS before wells were blocked with cell culture medium (200uL/well) at room temperature for 30 minutes. After this, irradiated EBV, T cell clones, and drug were added in identical concentrations/conditions as the previously described proliferation assay. Plated were then incubated for 48 hours at 37˚C and 0.5% CO_2.

Wells were then washed five times with PBS and 0.5% FBS before 100uL/well detection antibody (diluted as per manufacturer instruction) and incubated for two hours at room temperature. Plates were then washed five times with PBS and 0.5% FBS, at which point streptavidin-ALP (100uL/well, 1:1000 dilution) was added and incubated at room temperature for one hour. Again, plates were washed five times with PBS and 0.5% FBS before filtered BCIP development substrate was added to all wells (100uL/well) for 15 minutes in the dark. After fifteen minutes had elapsed the back-plate was removed, and the plate carefully rinsed under the tap to stop the reaction. Plates were left to dry for a minimum of 24 hours before being read on the ELISpot plate reader.

2.5.10 ELISA

ELISA kits were obtained from eBioscience and utilised in accordance with the manufacturer's directions. ELISA assays were used to quantify secretion of cytokines into the supernatant of our T cell assays. Briefly, a capture antibody was coated (at manufacturer's recommended concentration onto the wells of a 96-well flat bottomed cell-culture plate (along with HBSS and 0.5% FBS) and incubated overnight at 4 degrees Celsius in the dark. Wells were washed with 5x PBS and blocked for one hour with assay diluent. Samples (supernatant) was added (100uL/well) before being incubated for 2 hours at room temperature. Plates were washed before detection antibody was added (diluted to manufacturer recommended concentrations in assay diluent) and incubated for a further hour at room temperature. Plates were washed again before 100uL/well, 1ug/mL streptavidin-horseradish peroxidase was added to wells and incubated for 30 minutes. Wells were washed and 100uL/well TMB substrate solution was added to wells for 15 minutes. Stop solution (1M NaOH) was added 50uL/well, plates were read at 450nm.

2.5.11 RNA Isolation & Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total cellular RNA was extracted using TRI-Reagent (Sigma, USA) following the manufacturer's instructions. Briefly, RNA was resuspended in water and quantified using a NanoDrop 2000/2000c UV-Spectrophotometer (Thermo Scientific, USA). Using 100ng of total RNA, miRNAs were reverse transcribed using the Universal cDNA synthesis kit II (Exiqon, Denmark) using a thermocycler (42 degrees Celsius – 1 hour, 95 degrees Celsius – 5 minutes, finally, 4 degrees Celsius). The cDNA was mixed with ExiLENT SYBR Green Master Mix (Exiqon) and with specific primer sets for miR-9, miR-18a, miR-19b, miR-21, miR-146, miR-155, and miR-214. Quantitative PCR was performed using a ViiA 7 Real-Time PCR System (Thermo Scientific, USA) (95 degrees Celsius – 10 minutes, 10 cycles of 95 degrees Celsius – 10 seconds, 60 degrees Celsius – 1 minute).

The results of the qRT-PCR were calculated as relative expression (RE) by comparing the expression of drug treated cells with nontreated using U6 as a reference gene (as seen below). All qPCRs were performed in triplicate, and values higher than 1 were considered as an up regulation in the expression.

Calculation for RE values:

$\mathbf{\Delta\Delta CT} = \Delta CT_{24} - \Delta CT_{0}$

$\mathbf{\Delta\Delta CT} = (CT_{t(miR\text{-}155)} - CT_{t(U6)}) - (CT_{0(miR\text{-}155)} - CT_{0(U6)})$

$\mathbf{RE} = 2^{-\Delta\Delta CT}$

3 THE DEVELOPMENT OF A SCREENING ASSAY SYSTEM TO PREDICT THE INTRINSIC IMMUNOGENICITY OF A DRUG OR COMPOUND.

3.1 INTRODUCTION

In recent years, drug hypersensitivity reactions (DHRs) have become increasingly problematic for patients, clinicians, and the pharmaceutical industry. This has led to high morbidity and mortality rates for patients who suffer an adverse reaction from a prescribed drug. ADRs have provided an additional administrative and financial burden on the NHS in the UK; with projected costs being in the region of £500 million and 4% of the hospital bed capacity being utilised due to such reactions even 15 years ago [1]. The financial burden of post-licensing drug withdrawal due to DHR, in-part, contributes to the high cost of drug development for a single drugs exceeding $2billion. Hence proper diagnosis and prediction of DHR is of the upmost importance [234,235].

The diagnosis of the culprit drug in DHR can be difficult; especially in hypersensitive patients on multi-drug therapy when drug-drug interactions can cause even more confusion. Classification of the reactions can be aided by the time of onset of the reaction, as immediate reactions are often innate, mast cell mediated. Whereas delayed type IV hypersensitivity reactions have an onset greater than one hour after drug administration, as the mechanism of action has APC processing, interaction with the T cell TCR, and priming of naïve T cells a prerequisite for a response to occur [15]. The introduction of skin testing for the diagnosis of drug allergy has improved the ability to diagnose DHR. Some studies have determined that diluting the active principles in skin patch testing is the best way to detect delayed hypersensitivity to corticosteroids (rather than intradermal testing) [236]. Whereas others have stated that whilst one can achieve 72% positive skin tests for suspected DHR, a combination of patch, prick and intradermal tested dependent on the drug and clinical manifestation of cutaneous ADR should be observed [16]. In contrast to the findings of Barbaud *et al,* another (albeit

limited study) concluded that one must use a combination of skin testing, IgE determination, and drug challenge after observing only a 27% confirmation of reaction in patients with a history of beta-lactam hypersentivity; displaying none of the above tests to be robust enough to stand alone [237]. *In vitro* assays for DHR diagnosis are preferable to drug challenge/intradermal or provocation tests as they are less invasive for the patient and can be done without risk of further allergy. Upregulation of CD69 or CD203c as markers of basophil activation has also become a useful *in vitro* tool for the diagnosis of immediate DHR as it often has higher sensitivity and specificity than other *in vitro* tests, despite variance in results between different drug classes [238]. Basophil activation, along with lymphocyte activation test can increase the sensitivity of *in vitro* diagnosis, potentially obviating the need for more invasive traditional skin testing [239].

The lymphocyte transformation test (LTT) is a useful *in vitro* assay for the diagnosis of delayed-onset DHR as it can be used to detect drug triggered T lymphocyte proliferation in patient PBMC after 6 days exposure to the drug. Patient PBMC can be cultured with the suspected culprit drugs for 6 days, and then T lymphocyte proliferation can be measured by ^{3}[H] thymidine incorporation. Studies have previously shown that skin patch testing or the LTT can be used to confirm clinical diagnosis of DHR; with the LTT displaying a marginally improved success rate (6/10 vs 6/20) [240]. Although the skin tests displayed the same specificity as the LTT in some studies (85%), the sensitivity was lower than the LTT, meaning the LTT can be considered a more useful tool than skin tests in the diagnosis of delayed onset DHR [241], especially if one considers the risks to the patient and invasiveness of the respective techniques. Later studies have shown that flow cytometric evaluation of CD69 upregulation on T cells may be a more useful tool than the LTT as it overcomes the

main weakness of the assay. The 6-day assay time can be critical if one needs to rapidly evaluate which drug in a multi-drug therapy is the cause of the DHR, whereas CD69 upregulation can be observed in all of the LTT positive patients, but in a 2-day timeframe [242]. Yet more work must be done to improve the diagnostic assay systems currently employed as the LTT can be inconsistent. Detection of positive LTT in 15 SJS/TEN patients had a sensitivity of 27% in a Porebski *et al.,* study in 2013, whereas detection of granulysin expression alongside ELISpots for granzyme B and IFN-γ provided 80% sensitivity [243].

Despite some advancement in the development of DHR *in vitro* diagnostic tools, the prediction of DHR remains an inherent problem in the drug development process. As delayed onset DHR is idiosyncratic in nature, there is often no clear picture of the potential immunogenicity of a novel compound or drug during early development. The drug could be safe in the majority of patients, with only a very limited number with the potential of suffering DHR. Over the years there have been a plethora of hypotheses why some drugs cause an unwelcome immune response and DHR at therapeutic dose. It is thought that if the mechanisms and reasons for immune activation can be elucidated, then the ability to predict said events prior to drug approval will follow.

Genetic susceptibility factors such as HLA alleles have been outlined as a possible explanation for pre-disposition of certain individuals to DHR; and why a majority of patients do not have adverse immune responses to drugs. Genome wide association studies (GWAS) have shown links between certain HLA risk alleles with a greater susceptibility to DHR with specific drugs [128,145,244,245]. Unwanted T cell activation leading to iatrogenic disease arises from the drugs' direct interaction with a protein encoded by the specific risk allele. Both strong and weak associations have been

outlined, but despite extensive work in this field, the majority of carriers of the HLA risk alleles do not present with clinically defined DHR to a suspect drug. In fact, only HLA-B*57:01 has been proven to have a strong enough association with abacavir hypersensitivity syndrome to be introduced into pre-approval screening programmes before being prescribed to HIV patients [7]. Due to its NPV of 100%, incidence of 'probable or definite' response to abacavir treatment has dropped from 1.2% to 0.2% by 2015 [246]. Other HLA alleles have been identified as risk factors of drug hypersensitivity: HLA-A*31:01 with carbamazepine in Europeans [245], HLA-B*57:01 with flucloxacillin-induced liver injury [31,145], HLA-B*15:02 and carbamazepine in Han Chinese populations [244]. Of the above only HLA-B*15:02 in Han Chinese populations has been robust enough for clinical application as part of a cost-effective pre-screening strategy. Sulfamethoxazole (SMX - parent drug of nitroso-metabolite used in development of the assay in this chapter) had been thought to cause DHR in some HIV patients due to minor HLA allele changes, but no strong HLA allele associations were detected [133]. Viral infection, reactivation, or viral activated cross-reactivity has also been thought to be a risk factor for DHR [247,248]. But as many DHR patients do not display viral reactivation [249] there must be other factors at play. The elucidation of the pathomechanisms of T cell activation in response to drug treatment is a key determinant of whether or not we can successfully predict DHR moving forward. The naïve T cell priming assay allows for this [71], but some improvements must be made in order to create a clinically relevant, useful assay for the drug development process.

The naïve T cell priming assay makes use of isolated naïve T cells from healthy donors, which are then primed to a specific drug-derived antigen over a 14-day period in the presence of dendritic cells. Upon re-challenge with the specific antigen a number

of different readout assays can be used to detect drug-specific T cell activation; including T cell proliferation by ^{3}[H] thymidine incorporation and cytokine secretion in ELISpot assays [71]. The assay utilised by Faulkner *et al.,* has since been used to determine healthy donor naïve T cell priming when altering a number of factors. The assay has been used to determine the effect of immune check point inhibitors on DHR; including PD-1 and CTLA-4, (both of which are important signalling components and drug targets in cancer immunotherapy [250,251]) whereby blockade of the checkpoint inhibitors allow for increased naïve T cell priming and T cell proliferation, and re-introduction of T regulatory cells dampen the immune response [182]. This outlines the need for both signal 1 *and* signal 2 in the T cell activation cascade and brings to light some of the limitations of the original naïve T cell priming assay. Another limitation of the original assay is that whilst healthy volunteer naïve T cells can be successfully primed to SMX-NO, Bandrowski's Base, piperacillin and other class I restricted drugs; however, naïve T cells were unable to be primed to any drugs that display class II restriction [135]. The assay also fails to take into account the precursor frequency and T cell repertoire for a specific antigen; instead assuming the same across all drugs. However, due to the diversity of the T cell repertoire, naïve T cells specific for a peptide-MHC complex are rare [252] and can vary from drug to drug. Cell numbers can also be problematic in instances where high volumes of blood from donors are not available. For the original naïve T cell priming assay, we take 108mL blood in order to be confident in obtaining enough PBMC, and thus, naïve T cells and CD14+ cells for dendritic cell generation. Finally, the assay requires a 2-3 week period for the detection of T cell priming and drug specific T cell proliferation from one healthy volunteer blood donation.

In order for the naïve T cell priming assay to become a useful tool for pharma and the drug development process, it must be further optimised and enhanced to take into account all of the above, whilst successfully being able to determine the potential immunogenicity of drugs by way of drug naïve T cell priming and T cell proliferation on drug re-challenge. The ability to outline other mechanisms of T cell activation by means of cytokine secretion is also an important factor to consider in the development of a new assay.

This chapter will follow through with the first steps in the development of two new naïve T cell priming assays as part of a novel screening system for the potential immunogenicity of drugs. The first assay will be developed in order to determine the potential of a drug to activate the immune system using up to 16 donors on a single assay; the T cell multi donor priming assay (T-MDA). The T-MDA is higher throughput than the standard priming assay as it can screen multiple donors for immune responses to drugs at the same time and requires fewer cells than the standard priming assay. This enables the screening of multiple donors with different HLA risk alleles on a single assay. The second assay – the T cell multi-well assay (T-MWA) – can be used to investigate drug-specific immune responses in depth. The T-MWA will utilise forty wells per drug treatment, as opposed to triplicates in the standard assay. This ensures that the precursor frequency of naïve T cells for each drug will be taken into consideration. The T-MWA will also provide a scaling level of immunogenicity as opposed to the yes/no outcome of the standard priming assay. The assay will also allow for in-depth mechanistic evaluation of antigen-specific T cell activation as the cell numbers and number of assayed wells involved will allow T cell proliferation to be measured by 3H thymidine incorporation assays, whilst cell supernatant can be taken for ELISA assays to detect multiple cytokine secretion. Together, the T-MDA

and T-MWA are the first steps in the optimisation of the naïve T cell priming assay. This is a move toward a more comprehensive screening system to detect the immunogenicity of novel compounds and drugs; suitable for pharma and the drug development process.

3.2 CHAPTER AIMS

1. Develop a screening system to evaluate the intrinsic immunogenicity of a drug or compound.
2. The miniaturisation of the standard naïve T cell priming assay to increase throughput and ease of use of the assay.
3. The development of a medium throughput assay to screen multiple individual donor cells on a single plate in a single experiment.
4. The development of an assay that enables a deeper understanding of the immune response to drug treatment. This must determine the number of responding wells to drug challenge post-T cell priming that takes into consideration the precursor frequency of T cells and provide a scaling level of immunogenicity.

3.3 METHODS

All methods for techniques seen in this chapter can be found in detail in Chapter 2: Methods and Materials.

3.4 RESULTS

3.4.1 PRIMING OF NAÏVE T CELLS FROM 4 DONORS TO SMX-NO AND PIPERACILLIN IN THE STANDARD DENDRITIC-T CELL ASSAY

In order to be able to develop, optimise and improve the naïve T cell priming assay, it was important primarily to start this project by performing standard naïve T cell priming assays to drugs that would be used for the optimisation of the T-MDA and T-

MWA. Therefore, four distinct priming assays were performed to different healthy donors. Naïve T cells and CD14+ cells were isolated from healthy donors (which were differentiated and matured to moDC) and co-cultured with SMX-NO and piperacillin separately. After re-stimulation with each of the drugs, some donors elicited successful naïve T cell priming as drug-specific T cell proliferation was observed in some concentrations of the drugs; however, not all donors were successfully primed to each drug. Priming to SMX-NO was successful in all four donors as there was statistically significant increase in T cell proliferation ($p<0.05$) in at least one concentration of SMX-NO in all four donors. Contrasting this, piperacillin priming was successful in only one out of the four donors (figure 3.1.1), when there was a significant increase in T cell proliferation at 1mM concentration.

Figure 3.1.1

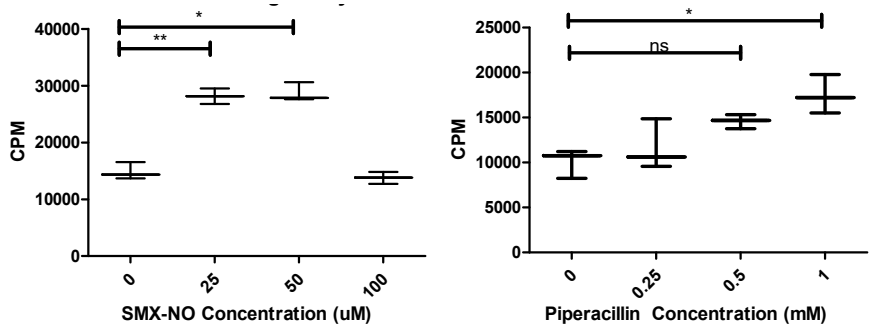


Figure 3.1.2

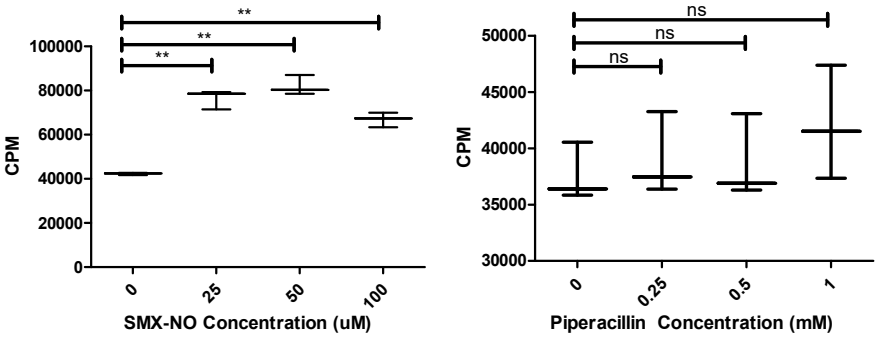


Figure 3.1.3

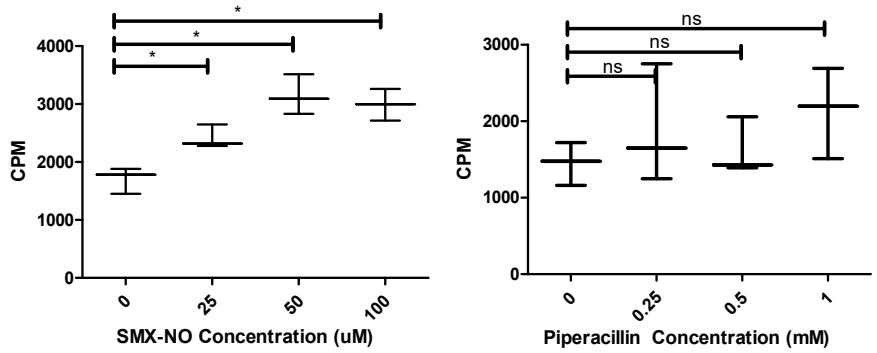


Figure 3.1.4

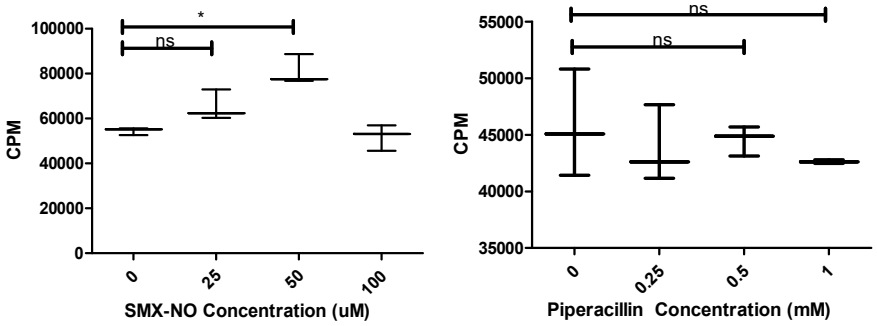


Figure 3.1.1, 3.1.2, 3.1.3, 3.1.4 *Priming of naïve T cells to SMX-NO and piperacillin using the standard priming assay*

*Naïve T cells ($2.5x10^6$ / well) obtained from healthy volunteers were co-cultured with autologous moDC ($8x10^4$ / well) with either SMX-NO (40μM) or piperacillin (2mM) for 14 days in a 24-well plate. T cells were then harvested and plated in a 96-well plate in triplicate ($1x10^5$ / well) with new autologous moDC ($4x10^3$ / well) to a range of SMX-NO and piperacillin concentrations and media as negative control. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Student's T test was performed to determine statistical significance of T cell proliferation at different drug concentrations compared to no drug treatment. Statistical significance was determined as; *$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$.*

Moving on from this, it was important to perform the first steps towards the miniaturisation of the assay, as the idea for the project was to keep all of the cells in the same plate from the start of the experiment until the end, as well as assaying as many wells as possible for each drug treatment. It was decided that SMX-NO would

be used primarily for the optimisation and development of the novel assay as it behaves as a model hapten; with positive priming of drug-naïve T cells detected in 9 out of 10 donors tested previously (Faulkner et al., 2012). For each of the healthy donor cells in figure 3.2, we assayed as many wells as possible from the cell numbers obtained from a single donation. SMX-NO priming to donor A resulted in the majority of wells responding to drug re-stimulation with T cell proliferation providing a stimulation index (SI) at least greater than 1.5. From the plate map and stacked column it can be seen that most of the responsive wells could be classified as good response (SI 2-3.99), and some strong responses (SI>4), with only two weak responses being observed. Donor B naïve T cells were primed to both SMX-NO and piperacillin. Again for SMX-NO more than half of the wells responded to SMX-NO re-stimulation with antigen-specific T cell proliferation generating most SI in the 'good' category, with equal numbers of weak and strong responses. In contrast, priming to piperacillin yielded a majority of negative SI (SI<1.5), with only one weak, and two good SI being observed.

3.4.2 Initial attempts at miniaturisation of the naïve T cell priming assay

It was also important to compare the standard priming assay to the developing miniaturised version of the assay, to ensure that T cell responses to drugs were consistent between both formats of the assay. Figure 3.3 displays the first miniaturised attempt at a T-MDA styled assay[B], which is compared to the standard priming assay [A]. In [B], cells were again cultured in the same 96-well plate from the start of the assay until completion. However, in this instance the cells were plated in triplicate as opposed to figure 3.2. As in figure 3.1, healthy donor naïve T cells were successfully

primed to SMX-NO, with antigen-specific T cell proliferation being observed at 40uM ($p<0.05$) and 80uM ($p<0.005$) in [A], and at 40uM ($p<0.005$) in [B].

1.85	8.95	6.23	3.88	0.37	0.70
4.13	4.81	2.01	3.96	4.30	2.34
1.67	1.42	2.04	3.67	0.65	2.35
7.88	0.94	3.19	1.01	0.64	1.45
2.78	3.19	1.11	0.84	0.78	0.11
0.14	0.32				

2.69	0.77	0.75	0.73	1.49	0.63
0.14	1.19	0.38	0.69	0.54	1.11
0.94	2.08	0.52	0.71	0.50	0.35
1.04	1.12	0.13	0.15	0.02	

2.24	2.31	5.67	0.62	6.16	2.47	1.12
1.62	1.51	3.22	4.09	0.72	2.55	5.44
1.20	2.48	1.31	2.08	1.15	0.96	1.97
0.45	1.99	1.07	2.30	0.60	0.60	4.91
1.99	1.90	3.14	2.13	4.14	1.38	0.41
0.35	1.38	0.79	1.39	0.74	0.60	2.74

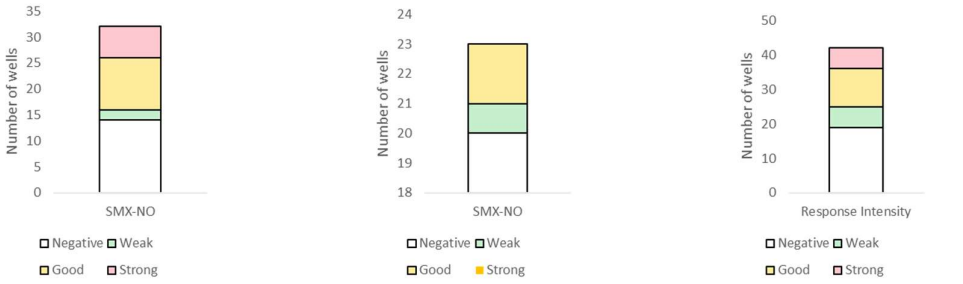


Figure 3.2 *Initial attempt at miniaturisation of naïve T cell priming assay*

Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40µM). After 14 days plates were centrifuged, and cells were washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40µM); multiple media treated negative control wells were also performed. After 48-hour incubation 3H-thymidine (0.5µCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Stimulation index was calculated as cpm drug treated well / average cpm negative control wells). The data was then displayed in a plate-map format, as well as stacked columns to show a traffic light system where; white = negative (<1.5 SI), green = weak response (1.5-1.99 SI), amber = good response (2-3.99 SI), red = strong response (>4 SI) based on scoring system in [71].

3.4.3 COMPARISON OF STANDARD SMX-NO PRIMING ASSAY AGAINST INITIAL ATTEMPT AT MINIATURISATION OF THE ASSAY

Upon optimisation of the T-MWA methods, as in figure 3.3, it was imperative to compare the assay to the standard priming assay. SMX-NO priming of naïve T cells was positive across all four donors (figure 3.5.1-3.5.4 [A]) to multiple concentrations of SMX-NO with statistically significant increases in antigen-specific T cell proliferation ($p<0.05$) compared to the control (no drug). The T-MWA [B] similar to the standard priming assay shows successful priming to all four donors. Box-plot analysis of [B] shows the spread of the data of the drug-treated wells compared to the

control as there are forty test wells of drug treated cells; potentially with different numbers of wells responding for each drug used and each distinct donor. Statistically significant increase in T cell proliferation of drug treated cells were observed compared to control wells in figure 3.5.1 ($p<0.05$), 3.5.2 ($p<0.005$), 3.5.3 ($p<0.0001$) and 3.5.4 ($p<0.0001$).

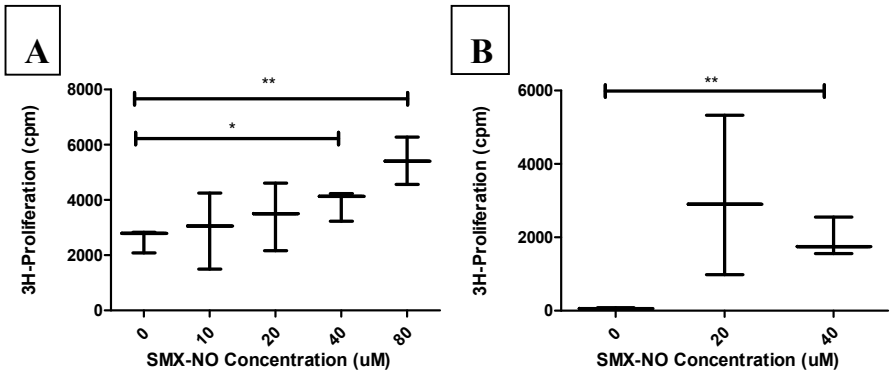


Figure 3.3 *Using SMX-NO to compare the standard priming assay to initial attempt of assay miniaturisation*

[A] *Naïve T cells ($2.5x10^6$ / well) obtained from healthy volunteers were co-cultured with autologous moDC ($8x10^4$ / well) with either SMX-NO (40μM) for 14 days in a 24-well plate. T cells were then harvested and plated in a 96-well plate in triplicate ($1x10^5$ / well) with new autologous moDC ($4x10^3$ / well) to a range of SMX-NO concentrations and media as a negative control. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Student's T test was performed to determine statistical significance of T cell proliferation at different drug concentrations compared to no drug treatment. Statistical significance was determined as; $*p \leq 0.05$; $**p \leq 0.005$; $***p < 0.001$.*

[B] *Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate in triplicate with autologous moDC (8000 / well) with SMX-NO (40μM). After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40μM) or media as a negative control. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Student's T test was performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug treatment. Statistical significance was determined as; $*p \leq 0.05$; $**p \leq 0.005$; $***p < 0.001$.*

3.4.4 T-MDA Assay – still in development – 5 Donor T-MDA assay to SMX-NO and Piperacillin

After comparing the standard priming assay to the developing T-MDA, the next focus would be to recruit multiple donors to plate in a single assay. The main strength of the T-MDA will be to identify drug-specific T cell responses in multiple donors in a single

plate, so that one can rapidly screen a number of different healthy volunteer cells; or potentially to multiple donors expressing a range of HLA alleles. In figure 3.4.1 5 healthy volunteer naïve T cells were primed to both SMX-NO and piperacillin in a single plate. Strong (donors 1,4 and 5 SI>4) or good (donors 2 and 3 SI=2-3.99) were observed in all five donors for SMX-NO priming, whereas for priming to piperacillin, only donor 2 elicited a strong response, with the others donors providing negative SI. In figure 3.4.2, SMX-NO priming was successful in all eight distinct donors, with four strong, one good, and three weak responses. Piperacillin priming was again mainly negative, with only two out of eight donors having positive responses to piperacillin re-stimulation; one strong and one weak response. Bandrowski's Base priming was was positive in four out of eight donors, with one strong, one good, and two weak responses.

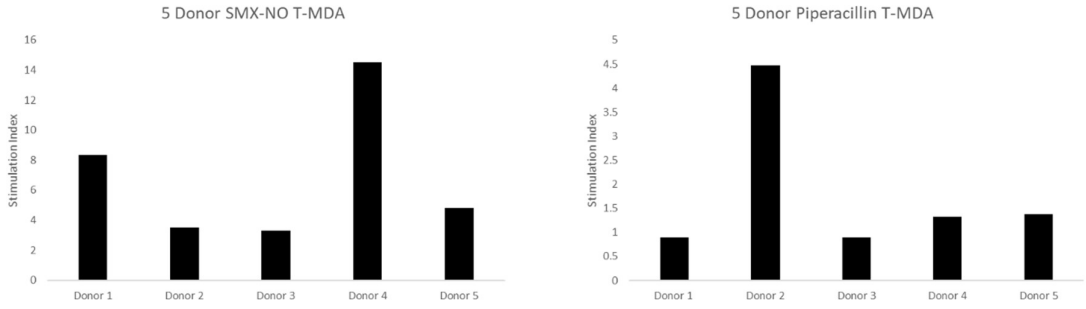


Figure 3.4.1 *T-MDA assay of five donors in a single plate assay*

Naïve T cells (2x10^5 / well) from five different healthy volunteers were plated in a 96-well plate in triplicate with autologous moDC (8000 / well) with SMX-NO (40µM). After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40µM), piperacillin (2mM) or media as a negative control. After 48-hour incubation 3H-thymidine (0.5µCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Stimulation index was calculated as cpm drug treated well / average cpm negative control wells). Negative (<1.5 SI), weak response (1.5-1.99 SI), good response (2-3.99 SI), strong response (>4 SI) based on scoring system in [71].

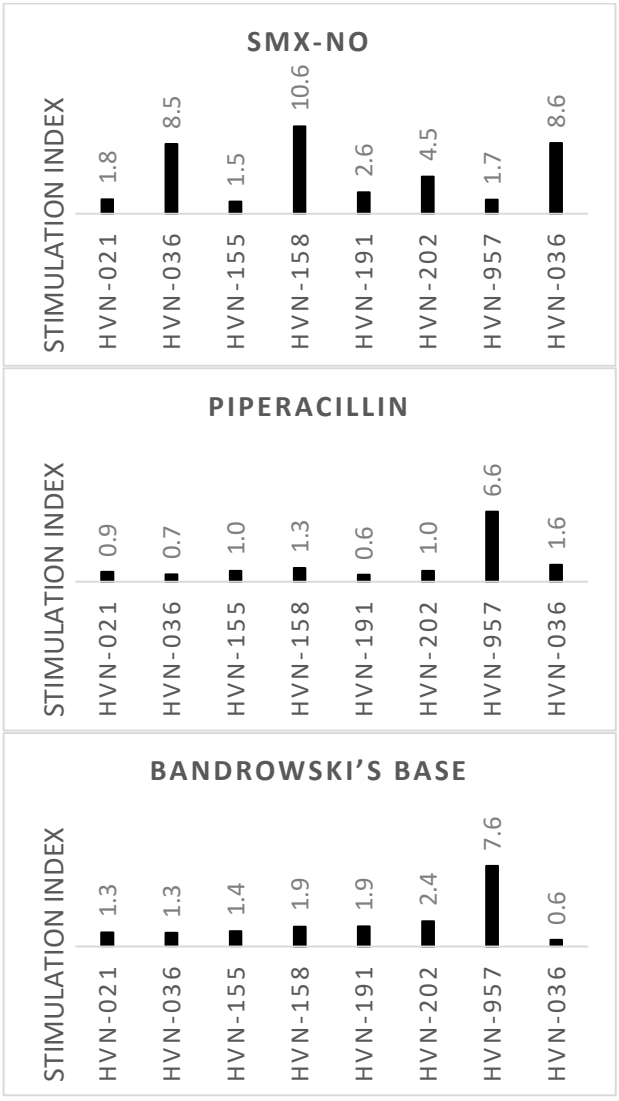


Figure 3.4.2 *T-MDA of eight donors in a single assay*

Naïve T cells ($2x10^5$ / well) from eight different healthy volunteers were plated in a 96-well plate in triplicate with autologous moDC (8000 / well) with SMX-NO (40µM). After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40µM), piperacillin (2mM), Bandrowski's Base (5µM) or media as a negative control. After 48-hour incubation 3H-thymidine (0.5µCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Stimulation index was calculated as cpm drug treated well / average cpm negative control wells). Negative (<1.5 SI), weak response (1.5-1.99 SI), good response (2-3.99 SI), strong response (>4 SI) based on scoring system in [71].

3.4.5 COMPARISON OF STANDARD PRIMING ASSAY AND T-MWA IN 4 HEALTHY DONORS

Upon optimisation of the T-MWA methods, as in figure 3.3, it was imperative to compare the assay to the standard priming assay. SMX-NO priming of naïve T cells was positive across all four donors (figure 3.5.1-3.5.4 [A]) to multiple concentrations of SMX-NO with statistically significant increases in antigen-specific T cell proliferation ($p<0.05$) compared to the control (no drug). The T-MWA [B] similar to the standard priming assay shows successful priming to all four donors. Box-plot analysis of [B] shows the spread of the data of the drug-treated wells compared to the control as there are forty wells of drug treated cells; potentially with different numbers of wells responding for each drug used and each distinct donor. Due to much lower background controls, statistically significant increase in T cell proliferation of drug treated cells were observed compared to control wells in figure 3.5.1 ($p<0.05$), 3.5.2 ($p<0.005$), 3.5.3 ($p<0.0001$) and 3.5.4 ($p<0.0001$). There is an observed difference in the background control CPM in all priming assays; this could be due to differences in background proliferation of T cells at different times as the immune system will be in different state of activation of responding slightly differently to nascent stimuli. This is why the calculation of SI and the use of the dot plot with statistical testing is important to decipher the meaning of the raw CPM values.

Figure 3.5.1

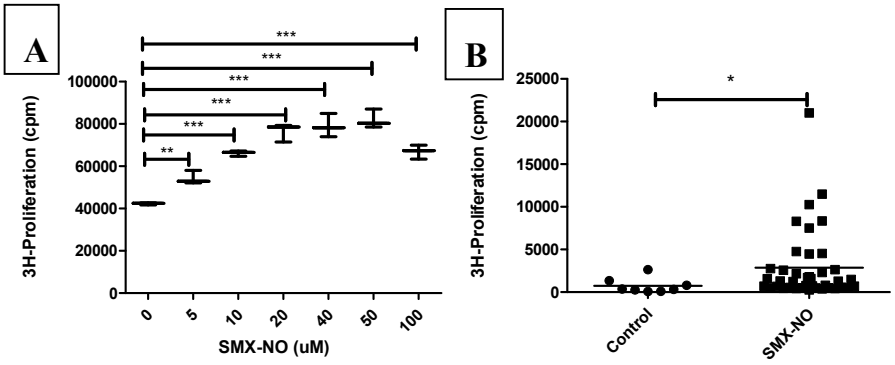


Figure 3.5.2

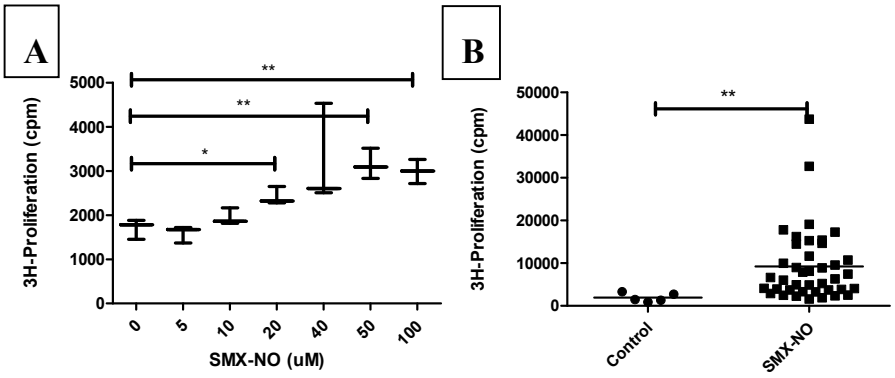


Figure 3.5.3

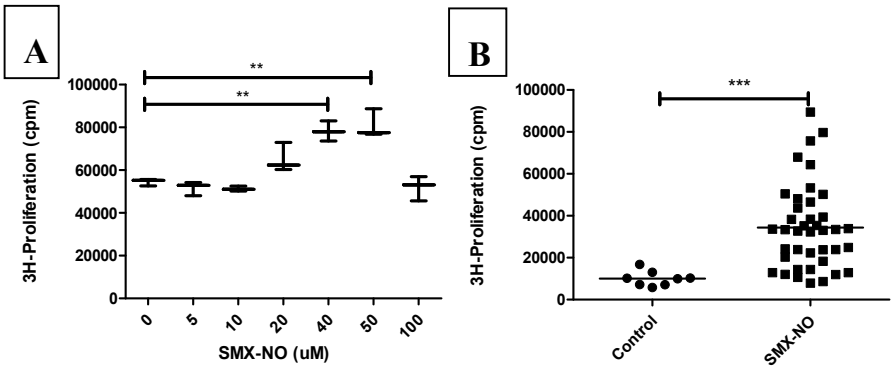


Figure 3.5.4

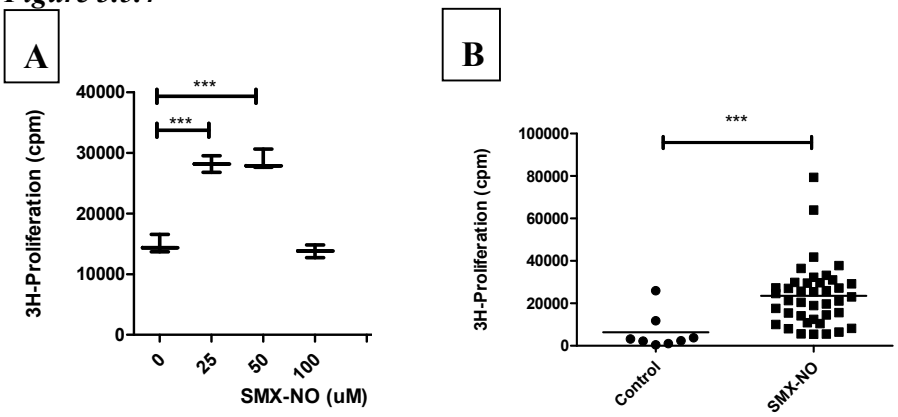


Figure 3.5.1, 3.5.2, 3.5.3, 3.5.4 *Comparison of standard naïve T cell priming assay and T-MWA in 4 healthy volunteers*

[A] *Naïve T cells ($2.5x10^6$ / well) obtained from healthy volunteers were co-cultured with autologous moDC ($8x10^4$ / well) with either SMX-NO (40μM) for 14 days in a 24-well plate. T cells were then harvested and plated in a 96-well plate in triplicate ($1x10^5$ / well) with new autologous moDC ($4x10^3$ / well) to a range of SMX-NO concentrations and media as a negative control. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Student's T test was performed to determine statistical significance of T cell proliferation at different drug concentrations compared to no drug treatment. Statistical significance was determined as; *$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$.*

[B] *Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40μM). After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40μM) or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Mann-Whitney test was performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug treatment. Statistical significance was determined as; *$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$.*

3.4.6 Reproducibility of the T-MWA – T-MWA assays to SMX-NO and piperacillin from two separate blood donations in two different donors taken six months apart.

For the T-MWA to be a viable assay for industry and the drug development process it is critical that the assay is reproducible in the same donor to ensure that the number of responsive wells remains the same or similar for the same donor from cells isolated from blood donations at different times. With this in mind, two separate donations were taken 6 months apart in two different donors and T-MWA assays were performed on the resulting isolated naïve T cells and cultured moDCs. In [A] SMX-NO resulted in mostly good response to drug re-challenge, with strong response observed the second most frequently in both donations. For piperacillin priming in [A], again the number and nature of response was similar across both donations, with mainly weak response seen in the positive wells. In donor [B] naïve T cells were primed to SMX-NO, again with similar numbers and nature of response seen between both separate donations. As in [A], most positive responsive wells were categorised as ‘good’, with fewer numbers of ‘weak’ and ‘strong’ response. As can be seen from the stacked column analysis, the overall number and nature of responses (negative, weak, good, strong) are similar between the two different donations for both donors tested.

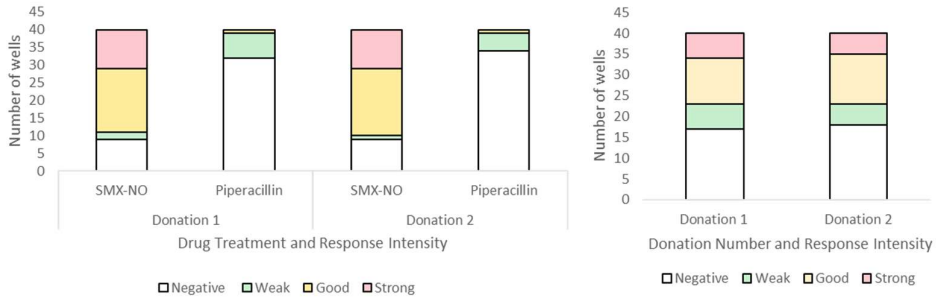


Figure 3.6 *Reproducibility of T-MWA results from 2 separate donations from the same volunteer*

Naïve T cells (2x10^5 / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40µM) or piperacillin (2mM). After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40µM), piperacillin or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation 3H-thymidine (0.5µCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Assays were repeated with the same donor, with blood donations taken 6 months apart. Stimulation index was calculated as cpm drug treated well / average cpm negative control wells). The data was then displayed in a plate-map format, as well as stacked columns to show a traffic light system where; white = negative (<1.5 SI), green = weak response (1.5-1.99 SI), amber = good response (2-3.99 SI), red = strong response (>4 SI) based on scoring system in [71].

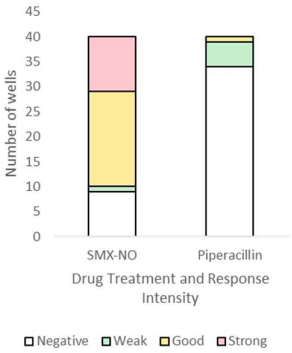
3.4.7 ASSESSMENT OF THE POTENTIAL IMMUNOGENICITY OF SMX-NO AND PIPERACILLIN ACROSS FOUR DONORS USING THE T-MWA

The optimised T-MWA was then utilised to determine the potential immunogenicity of piperacillin compared to our model hapten, SMX-NO. In figures 3.7.1 – 3.7.4, the plate map and stacked column traffic light system was used to determine the number of responsive wells and nature of the response in those wells. The scatter plot analysis was also performed in order to determine the spread of the total, raw proliferation data, and to determine if the antigen-specific T cell proliferation observed in the donors was deemed to be a statistically significant increase compared to the negative control (no drug). In figure 3.7.1 and 3.7.2 SMX-NO priming was successful in the majority of wells with responses mainly being categorised as good or strong, whereas piperacillin priming was mainly negative, with only a few weak or good responses but no strong responses. Overall SMX-NO priming was determined as successful with a statistically significant increase in T cell proliferation ($p<0.0001$ in 3.7.1 and $p<0.005$ in 3.7.2)

observed compared to control wells - T cell proliferation in response to piperacillin treatment was not statistically significant. Again, in figure 3.7.3, more positive responsive wells were observed after SMX-NO treatment than to piperacillin because of the large number of the non-responsive wells. Positive SMX-NO wells were mainly categorised as good or strong responses, with a smaller number of weak responses. Unlike in figures 3.7.1 and 3.7.2, strongly responding wells were present in the piperacillin treated wells, but these were fewer in number than the good and weak responses, with most wells being negative. SMX-NO treated wells resulted in a statistically significant increase in T cell proliferation, with piperacillin again being negative. In figure 3.7.4 supernatant was removed before addition of 3H-thymidine to perform ELISA assays for the detection of IFN-γ secretion with >50pg/mL IFN-γ classified as positive response. The number and nature of responding wells to both SMX-NO and piperacillin were again similar to those observed in previous 3.7 figures - again with SMX-NO response being determined to be statistically significant increase compared to control ($p<0.0001$) and piperacillin being negative overall. The ELISA did not match the proliferation plate map well for well, but the overall nature of response was conserved, with more responsive wells to SMX-NO than to piperacillin priming.

SMX-NO				
2.02	3.92	2.38	1.43	1.28
3.32	4.79	7.93	3.51	2.41
6.41	5.03	2.22	3.21	6.76
1.43	1.19	4.35	3.25	2.47
3.29	3.52	3.34	4.99	4.63
3.81	3.37	2.37	2.38	7.54
5.30	1.20	3.82	8.91	3.33
1.82	0.78	1.06	0.86	1.28

Piperacillin				
1.43	1.15	0.71	0.63	0.47
0.77	1.55	0.95	1.20	0.53
1.85	0.87	2.08	0.88	0.68
0.67	1.13	1.28	0.84	0.17
0.42	1.45	1.47	1.12	0.08
1.82	1.06	1.41	0.92	1.08
1.77	1.57	0.79	1.24	0.14
1.12	0.82	0.58	0.76	0.23



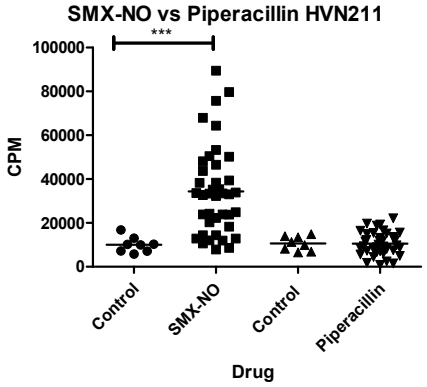
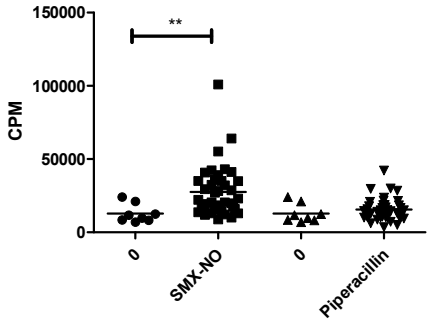


Figure 3.7.1

SMX-NO				
2.80	1.54	1.30	1.44	1.01
1.10	0.79	2.11	2.30	1.37
2.74	4.31	1.07	0.93	2.50
2.24	2.74	1.80	1.21	5.01
1.25	3.31	1.74	3.19	2.74
3.37	1.38	1.39	3.22	1.60
1.51	7.89	2.20	1.01	2.53
0.69	0.95	1.59	3.05	1.35

Piperacillin				
1.39	0.39	1.00	0.88	0.82
0.68	0.81	1.09	1.85	0.57
0.80	1.01	0.91	1.45	1.12
2.32	0.81	1.14	3.30	0.74
1.03	1.30	1.17	1.46	1.70
1.33	1.63	0.78	2.34	1.67
1.30	0.84	2.22	1.23	1.17
1.44	0.79	0.48	1.19	0.28



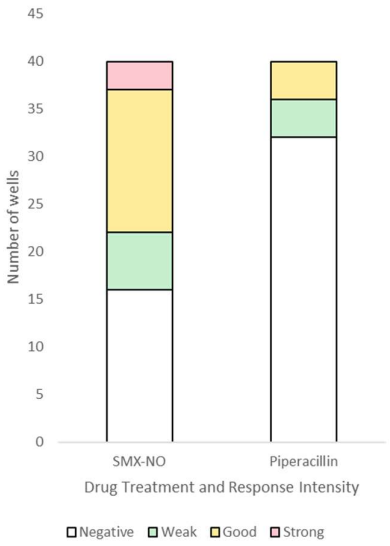
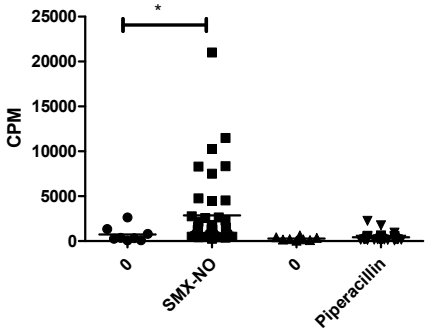


Figure 3.7.2

SMX-NO				
3.52	2.32	10.25	0.92	0.59
3.59	0.54	0.75	0.60	0.68
28.66	11.37	0.95	2.95	0.66
0.97	11.34	13.99	3.14	1.74
6.19	2.45	15.67	0.59	1.70
1.02	1.10	1.78	2.17	0.67
6.10	2.16	6.49	0.36	3.76
0.66	0.98	0.69	2.02	0.78

Piperacillin				
1.20	1.50	0.46	1.98	0.24
0.52	1.39	1.94	1.41	0.91
1.55	0.57	1.17	0.72	8.27
0.83	0.67	1.37	0.72	1.02
6.46	0.91	0.60	2.12	1.78
0.87	3.44	0.83	1.40	2.15
0.91	0.21	2.23	2.23	1.10
1.59	0.89	1.87	0.55	0.74



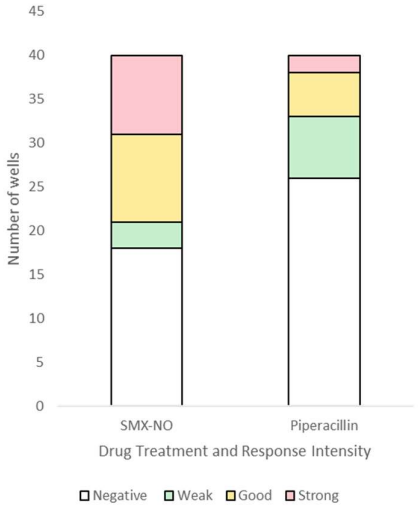
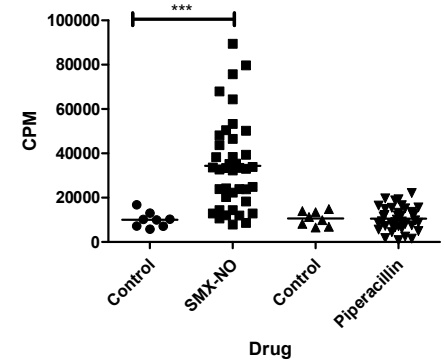


Figure 3.7.3

SMX-NO				
1.97	3.81	2.31	1.39	1.25
3.22	4.65	7.71	3.41	2.34
6.23	4.88	2.16	3.12	6.57
1.39	1.16	4.23	3.16	2.40
3.19	3.42	3.25	4.85	4.50
3.71	3.27	2.30	2.31	7.32
5.15	1.17	3.71	8.65	3.24
1.77	0.76	1.03	0.83	1.24

Piperacillin				
1.47	1.18	0.73	0.65	0.48
0.79	1.59	0.98	1.23	0.54
1.91	0.89	2.14	0.90	0.70
0.69	1.17	1.32	0.87	0.17
0.43	1.49	1.51	1.15	0.08
1.87	1.09	1.45	0.95	1.11
1.82	1.61	0.81	1.28	0.15
1.15	0.84	0.60	0.78	0.24

SMX-NO



Piperacillin

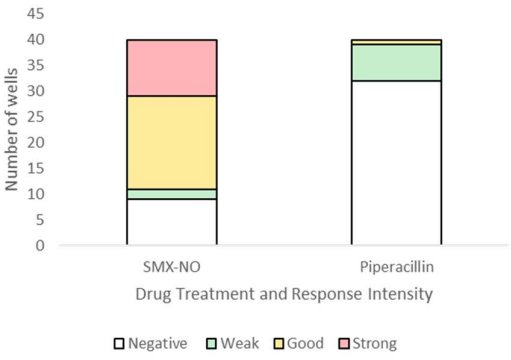


Figure 3.7.4

Figure 3.7.1 – 3.7.4 *Using the T-MWA to assess the potential immunogenicity of piperacillin compared to the model drug-hapten SMX-NO*

*Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40μM) or piperacillin (2mM). After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40μM), piperacillin (2mM) or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Stimulation index was calculated as cpm drug treated well / average cpm negative control wells). The data was then displayed in a plate-map format, as well as stacked columns to show a traffic light system where; white = negative (<1.5 SI), green = weak response (1.5-1.99 SI), amber = good response (2-3.99 SI), red = strong response (>4 SI) based on scoring system in [71]. Data was also plotted as scatter plot; Mann-Whitney test performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug treatment. Statistical significance was determined as; *p ≤ 0.05; **p ≤ 0.005; ***p < 0.001.* ***Figure 3.7.4*** *also had supernatant taken from the well after the 48 hour drug re-challenge and IFN-γ ELISA was performed according to Invitrogen assay instructions. Concentration of cytokine secreted in each well was determined before being plotted in plate format. Any red wells were deemed positive (>50pg/mL).*

3.4.8 Assessment of the comparative potential immunogenicity of SMX-NO, Bandrowski's Base and piperacillin across three donors using the T-MWA

Figure 3.8.1-3.8.3 shows naïve T cell priming data to SMX-NO, piperacillin and the contact allergen Bandrowski's Base in order than we could see the effect of using a contact allergen with a different mechanism of action to the other tested drugs/compounds. Priming of naïve T cells to Bandrowki's Base resulted in statistically significant increase in T cell proliferation upon primed T cell re-stimulation in all three donors ($p<0.0001$), with most or all wells showing positive response in all three donors. The nature of response seen was mainly good in figure 3.8.1 and strong in 3.8.2 and 3.8.3. SMX-NO resulted in Statistically significant increase in T cell proliferation ($p<0.0001$) in figure 3.8.1 and 3.8.3 but was not significant in 3.8.2. As in figure 3.7 the nature of positive responses in SMX-NO treated wells were mostly good or strong. Piperacillin priming was less successful than either BB or SMX-NO with mainly negative response wells in all experiments. Nevertheless, in figure 3.8.3 increase in T cell proliferation after priming to piperacillin was determined to be statistically significant ($p<0.05$).

SMX-NO				
2.37	2.98	0.93	1.35	2.75
0.76	2.35	0.51	3.07	3.86
1.75	0.97	1.43	1.82	1.95
0.60	2.27	2.13	0.74	2.52
2.52	0.52	1.63	2.76	2.40
1.97	3.49	1.89	3.37	1.01
2.70	2.50	1.44	2.73	5.91
0.51	2.87	1.15	7.34	1.30

Piperacillin				
1.11	0.49	0.08	0.66	0.04
0.91	0.93	0.28	0.26	0.41
0.34	0.83	0.46	0.38	1.23
0.59	0.45	3.56	0.55	0.24
1.84	1.29	2.30	2.09	0.34
0.58	4.56	0.79	0.32	0.33
0.59	0.80	1.26	0.50	0.72
0.29	0.16	0.33	0.13	0.08

Bandrowski's Base				
0.33	0.85	0.91	1.94	0.38
0.76	1.25	2.38	2.23	2.25
2.23	2.58	2.50	2.69	2.69
2.52	0.85	1.86	2.78	2.52
2.23	1.17	0.95	2.46	2.26
0.86	1.29	2.90	3.46	1.96
2.55	1.74	2.31	2.56	2.82
1.20	0.52	1.49	2.21	1.69

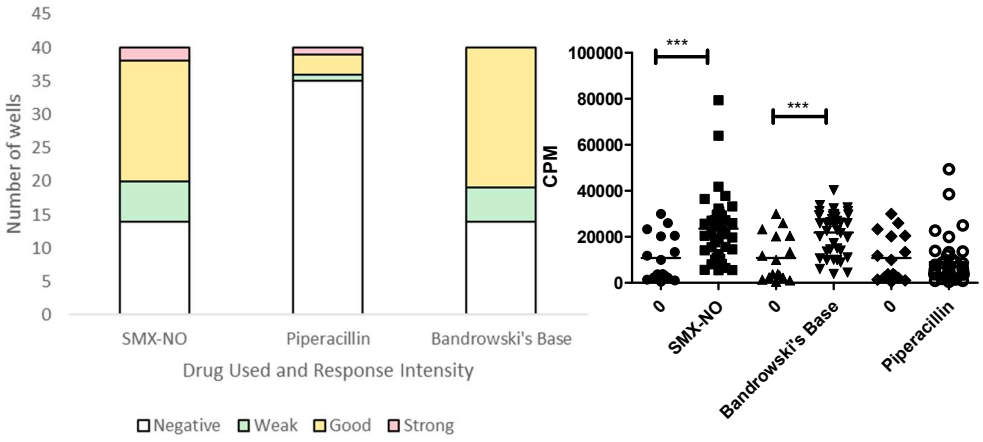


Figure 3.8.1

SMX-NO				
1.42	1.75	7.76	1.49	3.00
0.62	2.33	1.17	1.15	0.78
1.47	1.90	0.82	1.28	7.69
1.27	5.40	0.79	0.57	1.41
0.76	0.73	0.80	4.17	1.77
5.49	2.39	0.45	0.80	2.16
1.33	0.91	1.28	0.81	2.41
1.77	2.24	1.57	0.52	0.43

Bandrowski's Base				
4.43	3.49	9.06	5.32	7.96
10.69	9.86	5.64	12.04	9.16
6.28	4.72	4.96	11.98	16.67
6.17	6.24	5.85	3.59	5.40
4.81	9.01	3.07	5.39	6.46
9.99	9.33	5.68	12.01	34.35
5.11	16.50	7.36	19.27	8.06
10.85	5.77	2.13	5.38	7.02

Piperacillin				
1.34	1.55	1.35	1.48	1.51
1.38	1.69	1.61	0.86	2.24
0.78	0.66	0.67	1.60	1.38
1.27	0.73	1.08	0.99	1.41
0.49	1.47	1.30	1.31	1.42
0.51	0.95	1.30	1.65	0.76
0.79	1.09	0.55	0.66	0.82
0.69	0.59	0.85	0.87	0.47

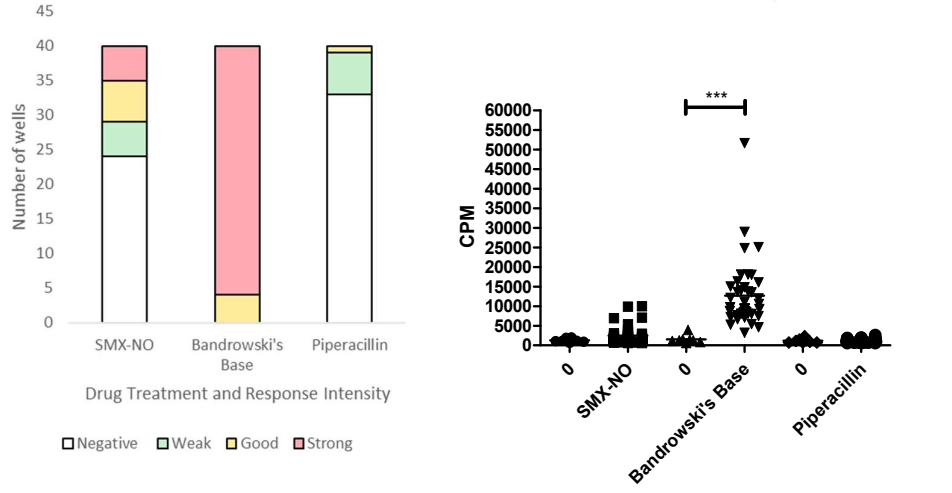


Figure 3.8.2

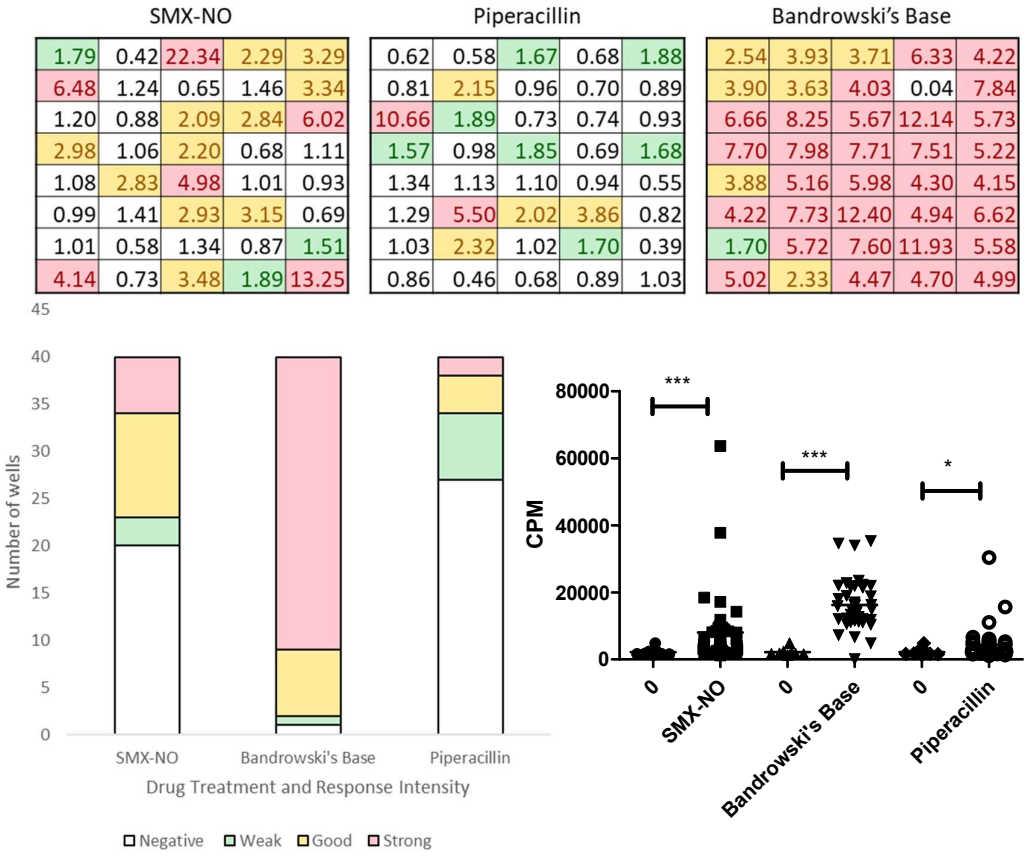


Figure 3.8.3

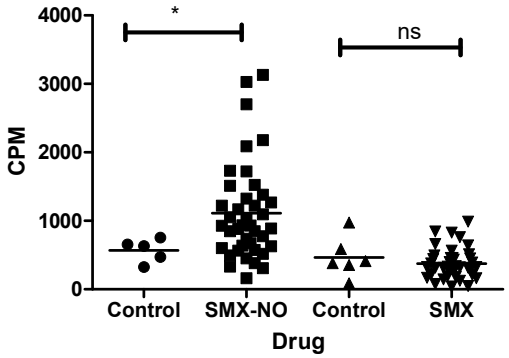
Figure 3.8.1, 3.8.2, 3.8.3 *Using the T-MWA to assess the potential immunogenicity of piperacillin compared to the model drug-hapten SMX-NO and contact allergen Bandrowski's Base*

*Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40µM), Bandrowski's Base (5µM) or piperacillin (2mM). After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40µM), Bandrowski's Base (5µM), piperacillin (2mM) or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation 3H-thymidine (0.5µCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Stimulation index was calculated as cpm drug treated well / average cpm negative control wells). The data was then displayed in a plate-map format, as well as stacked columns to show a traffic light system where; white = negative (<1.5 SI), green = weak response (1.5-1.99 SI), amber = good response (2-3.99 SI), red = strong response (>4 SI) based on scoring system in [71]. Data was also plotted as scatter plot; Mann-Whitney test performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug treatment. Statistical significance was determined as; *p ≤ 0.05; **p ≤ 0.005; ***p < 0.001.*

3.4.9 UTILISING THE T-MWA TO INVESTIGATE THE IMMUNOGENICITY OF THE REACTIVE METABOLITE NITROSO-SULFAMETHOXAZOLE, COMPARED TO ITS PARENT DRUG.

Finally, for this chapter, the T-MWA was used to determine the difference in priming of naïve T cells to SMX or its reactive metabolite SMX-NO. These experiments can outline the nature of the response and whether or not SMX-NO is more inherently immunogenic than its parent drug SMX. Across all three healthy donors (figure 3.9.1, 3.9.2 and 3.9.3) there are a higher number of responsive wells to SMX-NO than to SMX. Not only are there more overall responsive wells, but the nature of the response is generally stronger with higher SI seen across the plate. Many more 'good' responses are observed to SMX-NO than to SMX, whilst strong responses can be seen across all three donors, whereas only figure 3.9.2 displays SMX treated wells as having any strong responses. Box plot analysis of the SMX-NO data and subsequent statistical tests show that two of the three donors show a statistically significant increase in T cell proliferation (figure 3.9.1 $p<0.05$, figure 3.9.2 $p<0.005$). Priming naive T cells to SMX did not provide any statistically significant results in any of the donors tested. A positive response can be deemed as either a statistically significant increase in proliferation based on the dot plot, or a calculated $SI>1.5$ based on the 2012 paper which first developed the assay [71].

SMX-NO					SMX				
2.05	1.93	3.68	3.03	5.51	1.38	0.35	1.81	0.36	0.84
1.56	2.68	3.83	1.18	2.43	0.68	1.63	0.60	0.68	1.77
1.85	2.15	1.63	4.76	0.67	0.27	0.17	0.93	0.47	0.59
1.36	0.59	2.66	1.64	1.06	0.97	0.86	0.68	0.10	0.64
2.15	1.81	2.33	0.91	0.80	0.72	0.95	1.21	2.12	0.81
1.01	1.64	5.33	1.49	0.54	0.09	0.98	0.39	0.98	1.42
0.87	0.29	3.05	2.23	0.99	1.10	0.69	0.70	1.06	0.70
1.10	1.49	1.13	1.30	1.55	0.71	0.46	0.31	0.41	0.57



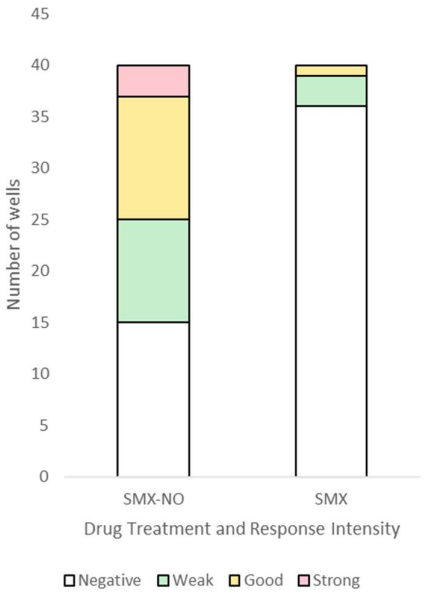
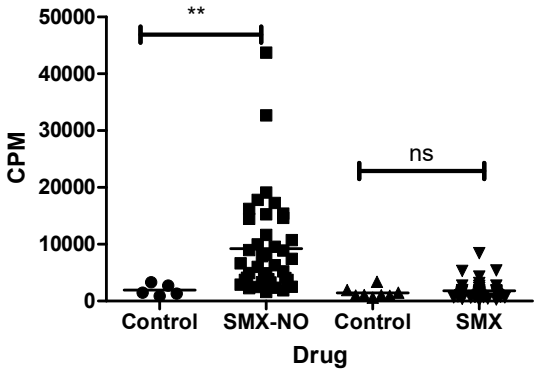


Figure 3.9.1

SMX-NO					SMX				
1.29	2.10	5.98	4.58	1.16	3.02	0.53	0.71	1.62	0.12
5.13	1.69	1.91	22.49	1.20	5.90	1.91	0.27	2.23	0.30
0.82	1.93	7.85	3.41	3.82	1.92	3.72	1.12	0.50	0.80
4.91	4.06	0.97	1.48	2.53	0.49	0.58	1.27	0.64	1.07
7.91	7.53	2.11	9.15	3.10	3.77	0.78	1.33	0.44	0.46
1.28	4.16	8.35	1.97	9.83	1.32	0.94	1.08	1.15	0.43
2.52	5.52	16.81	4.61	1.70	1.87	1.23	1.04	0.82	1.36
2.02	3.24	7.43	8.87	2.68	0.93	1.27	0.66	0.18	0.72



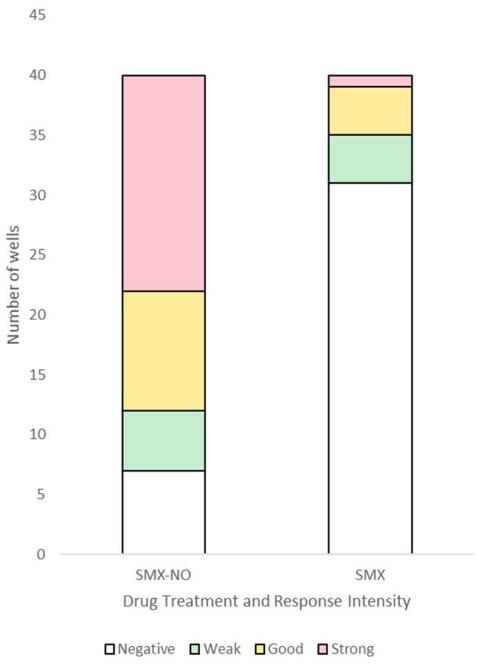


Figure 3.9.2

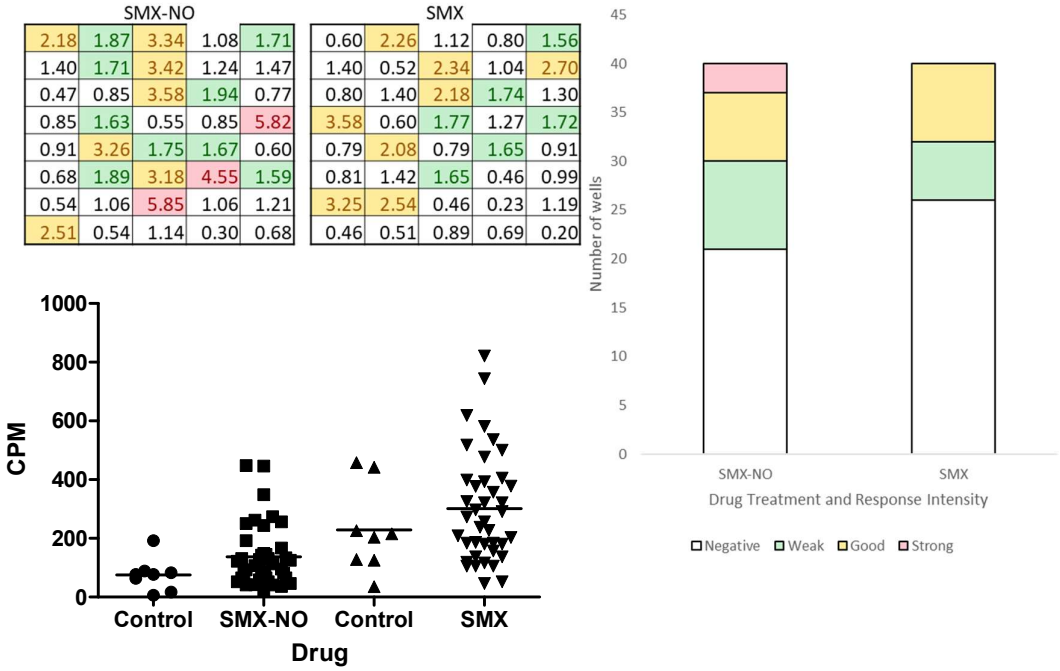


Figure 3.9.3

Figure 3.9.1, 3.9.2, 3.9.3 *Utilising the T-MWA to investigate the immunogenicity of the reactive metabolite nitroso-sulfamethoxazole, compared to its parent drug.*

*Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40µM), or SMX. After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40µM), SMX, or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation 3H-thymidine (0.5µCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Stimulation index was calculated as cpm drug treated well / average cpm negative control wells). The data was then displayed in a plate-map format, as well as stacked columns to show a traffic light system where; white = negative (<1.5 SI), green = weak response (1.5-1.99 SI), amber = good response (2-3.99 SI), red = strong response (>4 SI) based on scoring system in [71]. Data was also plotted as scatter plot; Mann-Whitney test performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug treatment. Statistical significance was determined as; *p ≤ 0.05; **p ≤ 0.005; ***p < 0.001.*

3.5 DISCUSSION

The development of a screening system to predict the intrinsic immunogenicity of drugs in development is becoming increasingly important for pharma and the drug discovery process. Drug withdrawal post-licensing is detrimental to both the drug industry due to large monetary loss, as well as the patients that lose the benefit of useful, generally safe drugs that cause severe DHR in a minority of patients. A non-invasive, medium/high throughput *in vitro* screening system will become critical for

detecting the potential immunogenicity of novel compounds in development, as well as determining the likelihood that an individual will develop a DHR to a licensed drug necessary for their treatment. HLA allele associations that determine predisposition to drug DHR have been a promising prospective tool for the prediction of DHR; purportedly to drugs such as carbamazepine, flucloxacillin and allopurinol [228,253]. Abacavir is one of the most well-known HLA associations that has been robust enough to be implemented clinically for pre-screening of appropriate treatment methods in HIV patients due to its 100% NPV. Recently, the use of skin explant models of DHR to determine the immunogenicity of drugs via histology on *in vitro* cultured tissues with predicted culprit drugs has showed some promise when combined with T cell proliferation assays and cytokine (IFN-gamma) assays [254]. Despite this, it would seem that this still does not define the mechanism of immune activation or provide any other mechanistic information on immune activation pathways (signal 1 / signal 2) such as the co-signalling pathways (PD-1, CTLA-4 etc.), as previous studies have determined their importance to immune activation in response to drug treatment [182]. This information could be important in the prediction of drug immunogenicity and could lead to similarities being observed between drugs with closely related mechanisms of immunogenicity. It also does not determine the likelihood that a drug can prime drug naïve T lymphocytes to invoke the observed immune response or provide any indication of relative scale of immune activation of various genetically and immunologically heterogeneous donors. The method is also only relative for drugs that cause cutaneous reactions, and as such, other culprit DHR drugs such as flucloxacillin, or drugs where we don't know what the manifestation of iatrogenic disease will be, cannot be tested using this system.

The standard naïve T cell priming assay has been used in various studies to determine the ability of a drug or antigen to prime drug naïve healthy donor T cells; which may give an indication of the potential immunogenicity of a drug. This assay can provide an avenue to determine the mechanistic inception of a DHR by T cell proliferation, as well as other phenotypic determination of the primed T cells through further T cell cloning investigations, cytokine secretion by ELISpot assays, and phenotypic analysis of T cell populations by flow cytometry. Nevertheless, the shortcomings of this assay mean that it is not yet ready for use by pharma to predict the intrinsic immunogenicity of a drug. In its current state, the assay lacks the propensity to screen multiple drugs or healthy donor cells on a single assay; and is not high throughput enough to be a viable method. The precursor frequency of the circulating antigen specific naïve T cells for the peptide repertoire of the expressed protein on the MHC surface is also not taken into consideration in the standard assay. It has been shown that the T cell precursor frequency in addition to antigen concentration is a determinant for whether an immune response will be mounted in response to drug treatment with different drugs. The likelihood of circulating T cells, specific to uniquely expressed peptide-MHC complexes exhibited by each drug will vary depending on the drug used, and as such, the likelihood that a drug will induce T cell response will vary accordingly [255–257]. The two new assays developed during this chapter; the T-MDA and T-MWA, attempt to rectify some of the issues associated with the standard priming assay and other common methods of the prediction of DHR.

The first figure of the chapter helps to explain the necessity of these changes, as the discrepancy between priming of naïve T cells to different drugs is clear. Only one of the four donors elicited a positive response to drug priming of both SMX-NO and piperacillin; whilst the other three donors were positive for SMX-NO priming, but

negative to piperacillin. DHR to piperacillin treatment occurs with a skin manifestation [258,259] and as such should be detectable in any predictive system employed. The assay must take into account that the precursor frequency of T cells varies with the drug in question, and that the likelihood of an immune response to DC:naïve T cell – drug culture will also vary according to the drug tested. A scaling level of immunogenicity could be employed in order to compare the relative immunogenicity of drugs against one another, compared to a model drug-hapten such as SMX-NO. Accompanying the above necessity is the laborious nature of the standard assay in which different cell types are cultured in different plates, the naïve T cell priming co-culture is then performed in a 24-well plate, before being moved to a 96-well plate for the readout stages. The miniaturisation of the T-MDA and T-MWA as described in the materials and methods section will aid both the ease of performing the assay, as well as enable the screening of multiple donors in a single 96-well plate. Or to have multiple wells (up to 40) per drug used which accounts for the T cell precursor frequency and the different levels of immunogenicity exhibited by different drugs.

Figure 3.2 was proof of principle that the miniaturisation was possible by performing the DC: naïve T cell – drug co-culture step in a 96-well plate, and then keep the assay in the same plate throughout the entire experiment without the need for addition of a second round of freshly generated autologous DCs (another limiting step for both time and resources; as more blood is needed for isolation of CD14s for DC culture). Successful priming was observed in a higher percentage of wells to SMX-NO than to piperacillin, in line with the status of SMX-NO as a model hapten/positive control. The nature of SMX-NO priming led to a more potent response with good and strong responses, than in piperacillin which were weak, with one good. Nevertheless,

miniaturisation of the assay was achieved as priming was observed in some of the wells to both drugs. Moving on from this the next steps would be to compare the standard priming assay against the T-MWA to ensure that at a minimum, instances of positive priming and T cell proliferation were mirrored by the T-MWA; for this SMX-NO was again used as the drug of choice for optimisation. The initial attempt to miniaturise the assay was successful as T cell proliferation was deemed significant in both versions of the assay (figure 3.3). Likewise figures 3.5.1-3.5.4 illustrates that the finalised version of the T-MWA (using 8 wells of control and 40 wells of drug treatments) results in positive priming of naïve T cells to SMX-NO; the idea now being that the T-MWA can be used to cover instances where standard priming was used previously, but will also be higher throughput, and more sensitive to use when testing other drugs or compounds.

The nature of the immune response observed by T cell priming to certain compounds in an individual should remain consistent. Any assay developed should be reproducible in determining the nature of the immune response to a drug challenge. To show that the T-MWA is not simply generating differing 'random' data each time an experiment is performed on a donor. This then allows the T-MWA to generate meaningful data when comparing a drug or compound's immunogenicity to a model allergenic hapten such as SMX-NO. For this reason, two blood donations were taken from two donors – with 6 months gap between each donation, ensuring that any T cell proliferation after drug challenge will be a consequence of the naïve T cell priming just taken place. Encouragingly, both the number of responding wells in the T-MWA, and the nature of the response (negative, weak, good, strong) remained consistent across both donations, in both donors, to all drugs tested.

The ability to screen multiple donors on a single assay for DHR is something that will enable quick determination of potential immunogenicity of novel compounds. This assay could work well in order to investigate potential changes in populations of people and may help elucidate why DHR occurs to drugs in some individuals, but not others. Donors of different ethnicities, or donors expressing differing HLA alleles could be screened using this method, which has previously shown to be effective in standard naïve T cell priming assays [32]. This could rapidly validate and indicate some of the risk factors associated with DHR to different compounds. Figures 3.4.1 and 3.4.2 help to outline this as multiple donors have been investigated in a single experiment. Ideally, up to 16 donors expressing unique HLA alleles can be assayed on a single plate, however, due to time constraints set by this project, as well as blood donation being a limiting factor, we managed to test 6 donors in the first experiment, and 8 in the second. As expected, different numbers of donors had positive priming responses to different drugs tested. SMX-NO, as expected, achieved positive SI scores in all patients across both experiments as it is a model drug-hapten that causes responses in almost all donors tested in previous work [136]. Whilst piperacillin achieved positive SI scores in 2 out of 5 donors, and 2 out of 8 respectively. Bandrowski's base achieved positive SI scores in only 4 out of 8 donors tested; something which was unusual in the context of our work, as previous work in the field has determined the contact allergen to be extremely allergenic. It is a product of auto-oxidation of para-phenylenediamine to an allergenic hapten causing contact dermatitis [135,232,260]. This could be due to cellular toxicity in the wells of the assay, resulting in lower proliferative responses, or indeed could be due to high background being observed in the negative control wells of the BB-primed T cells. BB produced T cell proliferation after priming categorised mainly as 'strong', which slightly contradicts data shown in

the T-MDA here. These differences could be due to cytotoxicity of strong sensitisers such as BB, which can have cytotoxic effects on dendritic cells in the culture due to inducible over-expression of CD86 surface marker [261].

Once established in our lab, it was necessary to assess different drugs using the T-MWA. As previously mentioned, SMX-NO and BB were used as model allergens, which should incite high levels of proliferative T cell responses after successful priming. Piperacillin was tested as a comparator drug. Previous studies have shown that PBMC stimulation with piperacillin does not result in positive LTT in healthy donors, but priming naïve T cells to piperacillin has resulted in positive proliferative response [32,135]. Results from the T-MWA, again presented piperacillin as less immunogenic than either SMX-NO or BB across all experiments. The number of wells responding to piperacillin was lower than SMX-NO and BB, whilst the nature of those responding wells was generally classified as weak responses, with some good responses. SMX-NO and BB responded in most (and some instances all) wells of the T-MWA, whilst the nature of the responses observed resulted in mostly strong, or good SI. No piperacillin responses were statistically significant. ELISA analysis was performed on the supernatant of the culture wells to correlate proliferation data with cytokine secretion from responding T cells. Whilst the cytokine data did not match with the proliferation data on a well-to-well basis, the nature of the response to the respective drugs was conserved in that fewer wells responded to piperacillin than SMX-NO. This data confirms that the T-MWA can be used to generate various data which can be analysed in a variety of ways to determine: the nature of the immune response by SI categorisation, a scaling level of immunogenicity dependent on the number of responsive wells, the spread of the data, statistical significance and cytokine secretion. Multiple ways of analysing the data generated from these assays must be

considered in order to determine the relative immunogenicity and likelihood of a drug inciting a DHR in a patient.

SMX hypersensitivity has been observed in antibiotic treatment options [248,262], however, the prediction of DHR to SMX has been difficult in previous experiments. Previous studies have shown that SMX induced DHR have been linked to metabolism of SMX; acting as a pro-hapten, firstly to a hydroxylamine, and then to its reactive metabolite form SMX-NO [110,263]. However, sulfamethoxazole has also been shown to activate T-cells directly with no requirement for metabolic activation[111]. We finally assessed the ability of the T-MWA to determine the relative immunogenicity of SMX, when compared to its reactive metabolite SMX-NO. Less than a quarter of the wells 4, 9, and 14 wells respectively registered as positive responses to SMX in the three donors, displaying a mixture of weak and good responses (and one strong), and no statistically significant responses. Again SMX-NO initiated statistically significant T cell response, with mainly good and strong SI responses. Although SMX can prime naïve T cells resulting in T cell proliferation in some wells of the T-MWA, this further confirms its reliance on metabolism to the reactive SMX-NO metabolite to cause strong responses *in vitro*. The T-MWA must improve upon this, as whilst detecting weak SI to parent drug could be helpful, the assay must be able to take into account those drugs that require metabolism to activate the immune system. SMX-NO is available for the study of SMX, but in instances where the reactive metabolite of a pro-hapten drug is not available or difficult to obtain; the addition of hepatocytes into a version of the T-MWA could be an effective tool in determining a drug's reliance on metabolism to exert its immune effects.

Whilst the main aims of the chapter were achieved by the miniaturisation of the standard naïve T cell priming assay through the successful development of two new

assays: T-MDA and T-MWA that provide a high-throughput system to investigate drugs that can potentially activate the immune system, more work is required to optimise these assays further before they can be considered robust enough for widespread use. Addition of hepatocytes to determine drug metabolism effects on likelihood of DHR would be a useful project. The assay in this chapter still hasn't investigated the deeper effects of signal 2 on immune activation. Co-inhibitory signalling pathways such as PD-1 and CTLA-4 in particular have been exposed as important in not only immune activation, but also the strength of observed T cell responses [75,182] and are becoming increasingly significant in the cancer field due to their use as immunotherapies for immune targeting of tumours [250,264]. It would be wise to also investigate the potential role of anti-inflammatory immune regulators in any assay being optimised. Differences in the number of T regulatory cells exerting anti-inflammatory effects such as IL-10 or TGF-β secretion could have an effect on observed immune activation if expressed differentially dependent on the drug used [182,265,266]. These issues with the two newly developed assays will be addressed in the following chapter.

4 INVESTIGATION OF IMMUNE REGULATION DURING NAÏVE T CELL PRIMING

4.1 INTRODUCTION

Type IV drug hypersensitivity reactions are unpredictable by nature; and cannot be pre-determined based on the pharmacology of the treatment drug. This is one of the major factors that cause DHR to be such a large (and increasing) problem in both the clinic and drug development.

Following GWAS studies HLA risk alleles have been considered an important determinant of whether or not DHR takes place [6]. Indeed, certain encoded proteins from HLA alleles in an individual interact specifically with a drug derived antigen for an immune take place. Many HLA risk alleles have been described in the literature, e.g. : HLA-B*57:01 and HLA-B*57:03 in flucloxacillin have been described as a causative factor in DHR and DILI in particular, whilst HLA-B*57:03 has been described as a risk factor in carbamazepine induced hypersensitivity, and abacavir is the most established drug with an associated HLA risk allele – HLA-B*57:01 [145,267]. Despite the extensive description of HLA risk alleles and their associated drug in the literature, most of the associations are not strong enough for compulsory screening to be implemented clinically.

Due to the relatively low PPV (positive predictive value – ability to correctly identify those with the ailment. Negative predictive value (NVP) is the ability to correctly identify those without the ailment) of many of the described HLA risk associations some other explanations have been put forward to describe why the majority of people who possess the one of the risk alleles do not develop DHR; and why DHR continues to be difficult to predict between different patient cohorts and individuals. Viral cross-reactivity whereby T cells activate the virus; or the virus mediates DHR directly has been proposed [247,268]. Comparably to the explanation of HLA risk alleles, viral cross-

reactivity does not provide an adequate explanation to these difficult questions as the majority of DHR patients do not present with viral reactivation [249].

In vivo and *in vitro* diagnostic and predictive tests have been developed to combat this, leading to correct diagnosis of the culprit drug for DHR and quick treatment, or even better, the prediction of a drug's potential immunogenicity and prevention of its use in patients. As shown in the previous chapter assays such as the DC: T cell priming assay and elucidate differences in T cell priming through T cell proliferation in response to treatment with different drugs. The drug used in the assay has a large bearing on the outcome of T cell priming and proliferation, thus the T-MWA was developed to more closely analyse the exact differences in T cell priming activation to upon different drug treatments; considering T cell precursor frequency.

In spite of the success of the different manifestations of the priming assay, the main question that now needs to be asked is are we recreating the correct immune microenvironment with all necessary immune regulation and parameters available, and can we modulate the immune regulation in order to truly elucidate the likelihood that a drug can invoke an antigen specific immune response.

Immune reactions require not only the formation of an immune synapse (MHC-TCR interaction), but a variety of other immune regulation pathways; termed signal 2. To be able to understand what is occurring at the cellular level in our screening systems we must also understand what forms of signal 2 are occurring in our assays, and whether we modulate these in order to further investigate their role in the initiation of undesirable immune activation in DHR. *In vivo* there is a balancing act that occurs for the initiation of immune responses between signal 1, and signal 2 that involve co-stimulatory and co-inhibitory signals that deviate between tolerance and activation.

A major component of signal 2 that has become increasingly important over the last few years are the co-signalling pathways that elicit their effects on T cell activation by modulating the threshold for activation. These comprise both co-stimulatory signals such as OX40, CD137, CD28 and ICOS [180], as well as the co-inhibitory pathways which include two of the focuses of this chapter PD-1 and CTLA-4 [174]. The co-inhibitory pathways PD-1 and CTLA-4 have become an important drug target for a variety of diseases; but their targeting and blockade by drugs in cancer treatment is of particular interest [174,178,269], whilst their role in drug-derived antigen-specific T cell activation and its associated complications needs to be investigated in more detail. The understanding of these pathways could be key to the success of the continued development of the T-MWA.

Tumour cells use the PD-1 pathway to evade host immune system; studies have shown there is a greater chance of death in cancer patients whose tumours express high levels of PD-1 [270]. Furthermore, differential expression of PD-1 on immune cells has been implicated in mediation of autoimmunity and whether or not an immune response proceeds; this gives credence to the idea that investigation into this pathway may help to mechanistically evaluate how T cells are primed, and immune responses are mounted both in patients that develop DHR, as well as in our *in vitro* systems. PD-1 is expressed on T cells, as well as a variety of other immune cells and interacts with its two ligands; PD-L1 which is similarly expressed across many immune cells types, and PD-L2, which has expression limited to DCs, activated macrophages, and some mast cells. Interaction between PD-1 and it's ligands can cause suppression of antigen-specific T cell response [186], whilst abrogation of the PD-1:PD-L1 axis can restore, or increase T cell response [137,182] through TCR coupling and dephosphorylation of TCR signalling; disrupting the MHC-TCR interaction.

CTLA-4 has also been characterised as an important immunoregulatory factor and another component of co-inhibitory signalling alongside PD-1. CTLA-4 is defined as a specific regulator of T cell activation, indeed CTLA-4 deficient (knockdown) mice often die within weeks of birth due to widespread lymphoproliferative disorders. As a component of co-signalling, CTLA-4 competes with the co-stimulatory CD28 for CD80 and 86 (its two ligands). As CTLA-4 expression is lower than CD28, its affinity for the two ligands is greater than CD28. Competition with CD28 for the two ligands can determine the likelihood of the development of an immune response, especially as CD86 has been characterised as co-inhibitory, whereas CD80 has been described as predominantly co-stimulatory [191]. CTLA-4 can also regulate immune responses through its effects on Treg-APC binding competition; as Treg binding to APCs inhibit the formation of a co-stimulatory immune synapse, in addition to Treg immunosuppressive effects [58,59].

Expression of the co-inhibitory components PD-1 and CTLA-4 may have a dramatic effect on naïve T cell priming and the outcome of our assays due to their immune-suppressive effects; and may help explain differences in naïve T cell priming and proliferative outcomes between different drugs used. Its is firstly important to determine the expression of PD-1 and CTLA-4 during the course of our priming cultures and determine if there are observable differences dependent on the drug used – we will use flow cytometry to determine cellular expression as well as overall expression in our priming culture of DCs, naïve T cells, and drug. Once we have observed the modulation of these pathways in our experiments, we can then functionally understand their importance through blockade of the pathways in our T-MWA assay (utilised in chapter 3). When comparing T cell priming without block to with co-inhibitory blocks, we can observe differences in priming and the functional

effects of co-signalling in our systems. This in turn can help in the further investigation of co-signalling relevance in DHR.

When discussing immune regulation alongside signal 2 in the context of DHR *in vivo* as well as in our predictive systems it is important to note the importance of Tregs. As we need to determine the viability of the immunoregulatory microenvironment we are creating in our assay, the presence of Tregs is important due to their immunosuppressive effects on T cell activation and proliferation which takes multiple forms. Tregs become increasingly relevant to DHR and our predictive *in vitro* systems when considering their aforementioned relationship with CTLA-4, as CTLA-4 expression can lead to increased Treg-APC interaction; causing lymphoproliferative suppression as a viable immune synapse cannot be formed. Tregs have also been implicated in IL-2 uptake – a key interleukin involved in T cell activation – which limits availability to, and subsequent activation of other T cells. This is also important as IL-2 has also been shown to induce Treg development and so despite its role in T cell proliferation activation of effector T cells, can also induce immunosuppressive events [60]. The secretion of the anti-inflammatory cytokines IL-10 and TGF-β from Tregs also form a large part of their immunosuppressive role in the immune system; again, through presumed disruption of APC antigen presentation to circulating TCR.

Induced Tregs (iTregs) may be generated from the naïve T cell population during priming, proliferating further and eliciting suppressive effects in our assay. iTregs are induced from a naïve CD4+ precursor and are usually supplemental to natural Tregs (nTregs). iTregs can elicit immune-suppressive effects and limit T cell activation, in this instance, TGF-β could be secreted from the DCs in our assay which can induce production of iTregs; something described in the literature [271,272]. We will investigate whether Tregs are generated in our priming cultures differentially dependent on the

treatment drug; as this may explain differences and difficulties observed in naïve T cell priming between different assayed drugs.

The knowledge gained from the investigation into immunoregulatory effects of co-signalling/signal 2 (specifically PD-1, CTLA-4 and Tregs) can help us understand the role and importance of immune regulation in cases of DHR, and can further help in the optimisation of predictive screening systems implemented in our research.

4.2 CHAPTER AIMS

1. Determine the Treg abundance in naïve T cell priming cultures, and determine any differences observed dependent on the antigen used for stimulation of the culture.
2. Determine the expression levels of co-inhibitory PD-1 and CTLA-4 in naïve T cell priming cultures, and determine any differences observed dependent on the antigen used for stimulation of the culture.
3. Determine the functional effects of PD-1 and CTLA-4 in our naïve T cell priming assays through blockade, and determine any differences observed dependent on the antigen used for stimulation of the culture.

4.3 METHODS

All methods for techniques seen in this chapter can be found in detail in Chapter 2: Methods and Materials.

4.4 RESULTS

4.4.1 GENERATION OF INDUCED TREGS (ITREGS) IN A NAÏVE T CELL PRIMING CO-CULTURE

In order to investigate the role of signal 2 and other determining factors in the regulation of the micro environment of T cell activation – such as the action of T regs

and co-signalling pathways (PD-1, CTLA-4) – it would be imperative to detect their presence in our naïve T cell priming cultures.

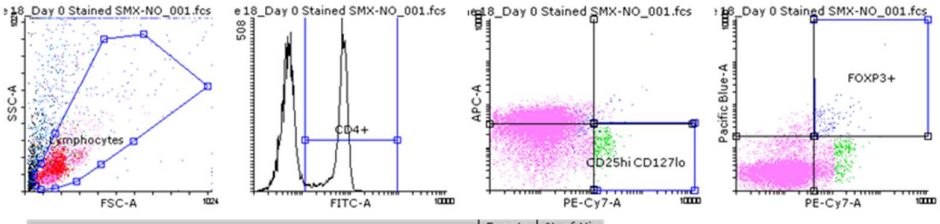
As our priming cultures in the T cell priming assay, T-MWA and T-MDA contain a cocktail of solely moDCs, naïve T cells, and drug, we needed to determine the presence of T regs as they were potentially generated from the naïve population. It was decided the best way to achieve this would be to take aliquot of cells from the priming culture at four different time points, with Tregs then being quantified by flow cytometry (Tregs determined as CD4+, CD25hi, CD127lo, FOXP3+).

Figure 4.1 shows three priming cultures from different healthy volunteers established through co-culture of moDCs, naïve T cells (isolated from PBMC) and drug (either SMX-NO or piperacillin). In all three instances the number of Tregs (as a percentage of CD4 cells) increased overall from day 0 to day 14 in response to treatment with either drug. In Treg 1 the peak number of Tregs occurred at day 14 to both drugs, with Tregs increasing from <0.5% at day 0, to 3% in response to SMX-NO treatment, and 2% in response to piperacillin treatment at day 14. In Treg 2 there was again an overall increase in Tregs from day 0-14 to both drugs. Piperacillin increase throughout the time points from 0.1 to 1.5% by day 14; whilst SMX-NO generated Tregs peaked at day 7 (0.1 to 2.4%), before decreasing to under 2% on day 14.

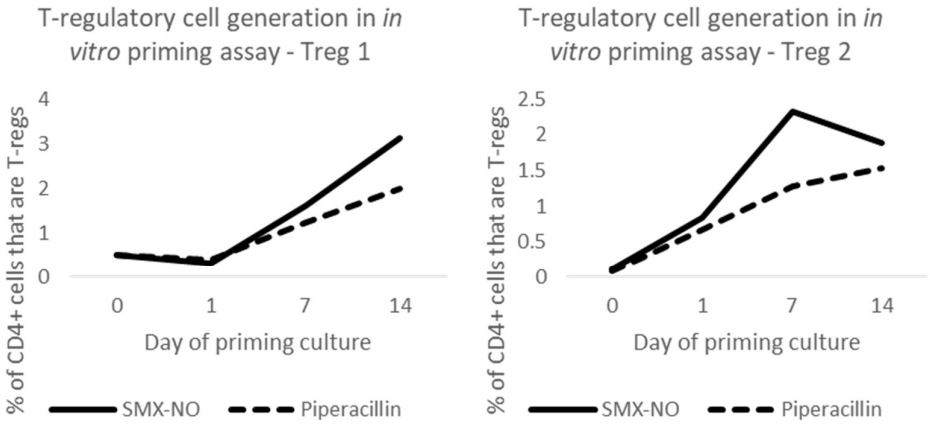
Treg 3 showed a similar story to Treg 2 in that there was an overall increase in Tregs in response to both treatments; piperacillin increased steadily throughout the time course to peak at day 14 (0.2 to 2.2%); whilst again Tregs to SMX-NO treatment peaked at day 7 (0.2 to 2%), before declining to day 14 1.5%. In two out of the three cases; SMX-NO treatment yielded a greater increase in Treg population than piperacillin.

4.4.2 SKEWING OF NAÏVE T CELLS TO A CD4/8 PHENOTYPE DURING PRIMING

After determining the generation of Tregs in the previous experiment; it was decided to check the skewing of T cells during the course of priming and whether the naïve T cells move towards a CD4/CD8 phenotype during priming (*figure 4.2)*. Three healthy donor priming cultures were set up and analysed as in the previous Treg experiments, but cells were stained and analysed for only CD4 and CD8. In all three instances, there were more CD8 cells than CD4 for both drug treatments – in all three instances the number of CD8 cells either decreased or remained constant in response to both drug treatments. In TC2 and TC3 the number of CD4 cells remained steady throughout the 14-day time course; with a slight decrease observed in response to piperacillin treatment in TC3. Whilst TC1 was interesting in that the number of CD4+ T cells increased overall by 15% by day 14.



	Events	% of Vis
All events	13,575	100.00
Lymphocytes AND CD4+	13,575	100.00
Lymphocytes AND CD4+ AND CD25hi CD127lo	231	1.70
FOXP3+	107	0.79
Lymphocytes AND CD4+ AND CD25hi CD127lo AND FOXP3+	16	0.12
Lymphocytes AND CD4+ AND CD25hi CD127lo AND FOXP3+ AND H-8	16	0.12
Left Bottom	12,391	91.28
Right Bottom	930	6.85
Left Top	141	1.04
Right Top	113	0.83



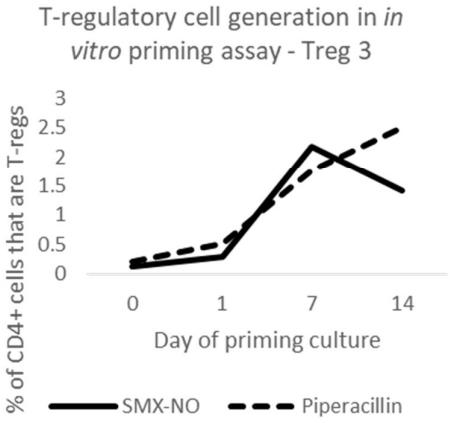
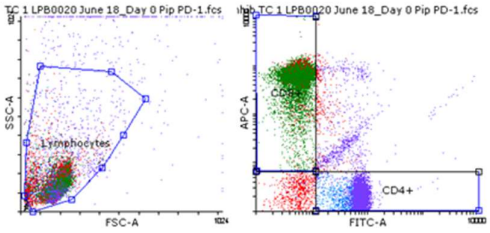
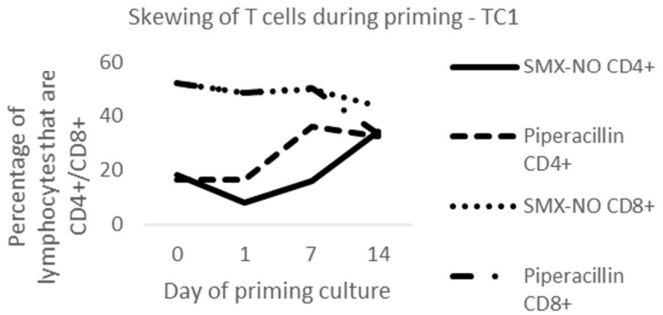
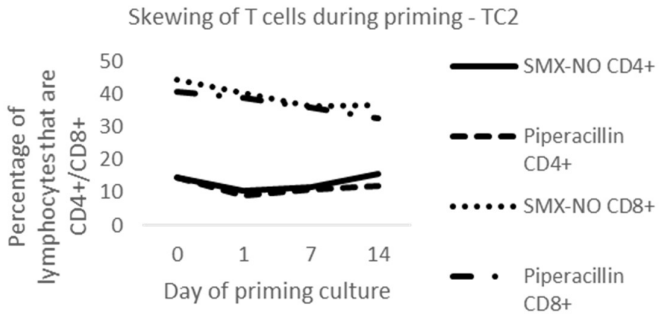


Figure 4.1 *Generation of Tregs during T cell priming - Naïve T cells (2.5x10⁶ / well) obtained from healthy volunteers were co-cultured with autologous moDC (8x10⁴ / well) with either SMX-NO (40μM) or piperacillin (2mM) for 14 days in a 24-well plate. T cells were then harvested on days 0, 1, 7 and 14 and stained with cell surface marker antibodies against CD25 and CD127. Cells were then fixed, permeabilised and intracellularly stained for FOXP3. Cells were analysed by flow cytometry on a BD FACS CANTO II. Gating strategy can be seen above whereby lymphocytes that were: CD4+, CD25hi, CD127lo, FOXP3+ were determined to be Tregs. % of CD4+ cells that were Tregs were determined at each of the four time points and displayed in the graphs above to determine generation of Tregs from an naïve T cell population during priming.*



	Events	% of Vis
All events	31,363	100.00
CD4+	5,224	16.66
Lymphocytes AND CD4+	5,224	16.66
CD8+	16,283	51.92
Lymphocytes AND CD8+	16,283	51.92
Lymphocytes AND CD4+ AND PD-1/CTLA-4	1,416	4.51
Lymphocytes AND CD8+ AND PD-1/CTLA-4	864	2.75
Left Bottom	1,056	3.37
Right Bottom	12,928	41.22
Left Top	16,305	51.99
Right Top	1,074	3.42





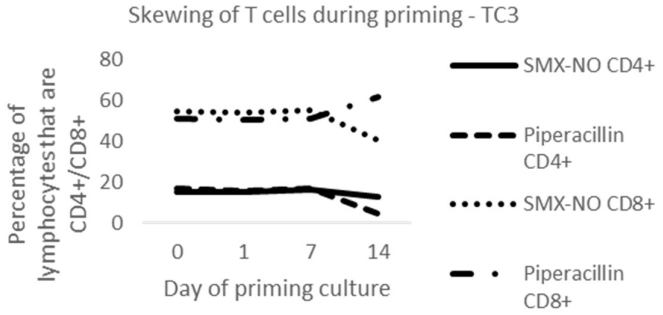


Figure 4.2 *CD4/CD8 skewing of T cells during naïve T cell priming - Naïve T cells ($2.5x10^6$ / well) obtained from healthy volunteers were co-cultured with autologous moDC ($8x10^4$ / well) with either SMX-NO (40μM) or piperacillin (2mM) for 14 days in a 24-well plate. T cells were then harvested on days 0, 1, 7 and 14 and stained with cell surface marker antibodies against CD4 and CD8. Cells were analysed by flow cytometry on a BD FACS CANTO II. Gating strategy can be seen above.*
% of lymphocyte cells that were CD4/CD8 were determined at each of the four time points and displayed in the graphs above to determine skewing of T cells from CD4/CD8 during the course of priming.

4.4.3 EXPRESSION OF THE CO-INHIBITORY CHECKPOINTS DURING NAÏVE T CELL PRIMING TO SMX-NO AND PIPERACILLIN

Following analysis of the CD4/CD8 expression on T cells during T cell priming culture we decided to analyse the expression of PD-1 and CTLA-4.

PD-1 and CTLA-4 expression was measured by flow cytometry in a similar experimental design and time course as the Treg and skewing experiments; only this time cells were stained for CD4, CD8, and then PD-1 or CTLA-4.

It was imperative to determine the overall expression of PD-1/CTLA-4 in the cultures throughout priming as there could be differential expression of different receptors; therefore, MFI would give an idea of overall intensity of expression of the co-inhibitory markers. In both donor 1 and 2 (*figure 4.3)* there were marginal increases in overall expression of both PD-1 and CTLA-4 on CD4 cells in response to both drug treatments; particularly from day 0-7. PD-1 expression of the CD8 population decreased in donor 1 but increased in donor 2 during the time course to both drugs, but CTLA-4 expression in CD8 cells marginally increased in response to SMX-NO treatment and decreased in response to piperacillin treatment in donor 1. In donor 2, CD8 cell PD-1 *and* CTLA-4 expression increased to a similar level in response to both SMX-NO and piperacillin treatment.

In donor 3, increases were seen across the board in both CD4 and CD8 cell expression of PD-1 and CTLA-4 in response to either drug treatment; with the greatest increases seen in response to SMX-NO treatment. Overall the greatest increase in PD-1 and CTLA-4 expression in both CD4 and CD8 T cells was observed in cells treated with SMX-NO.

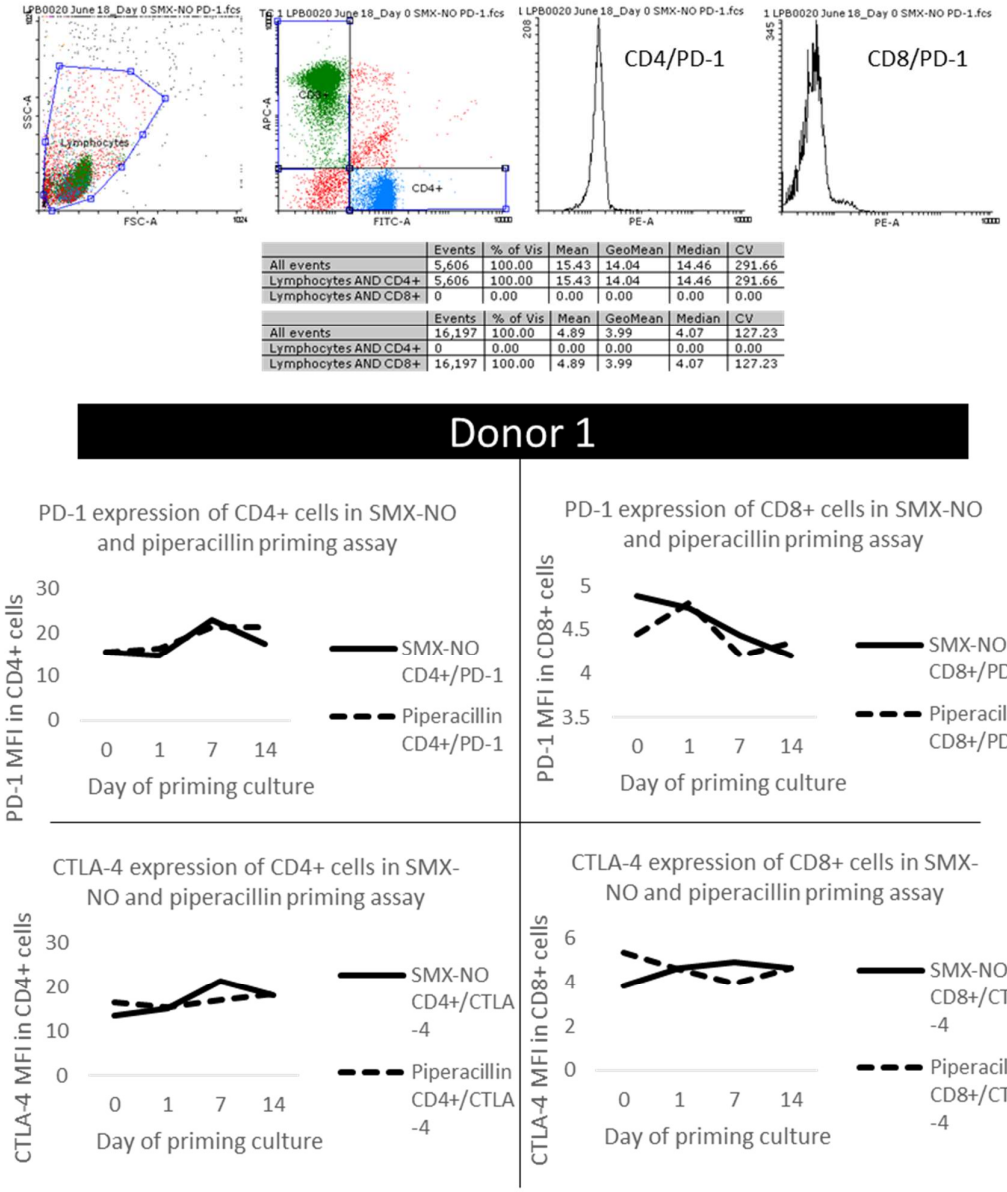
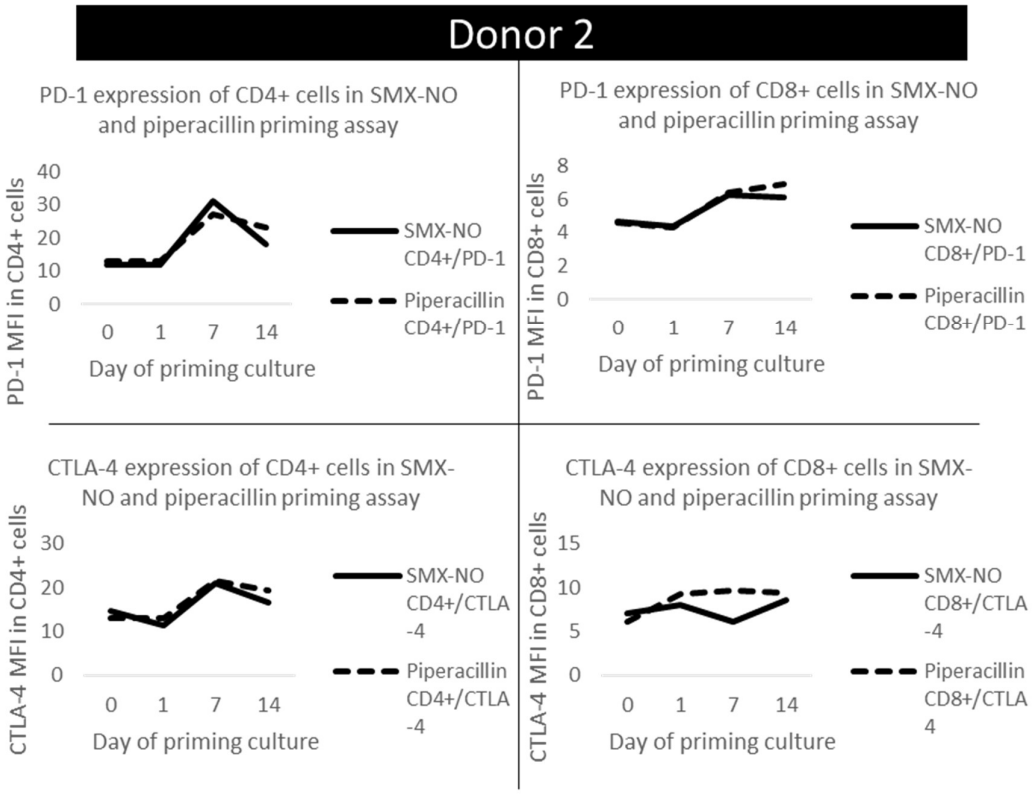
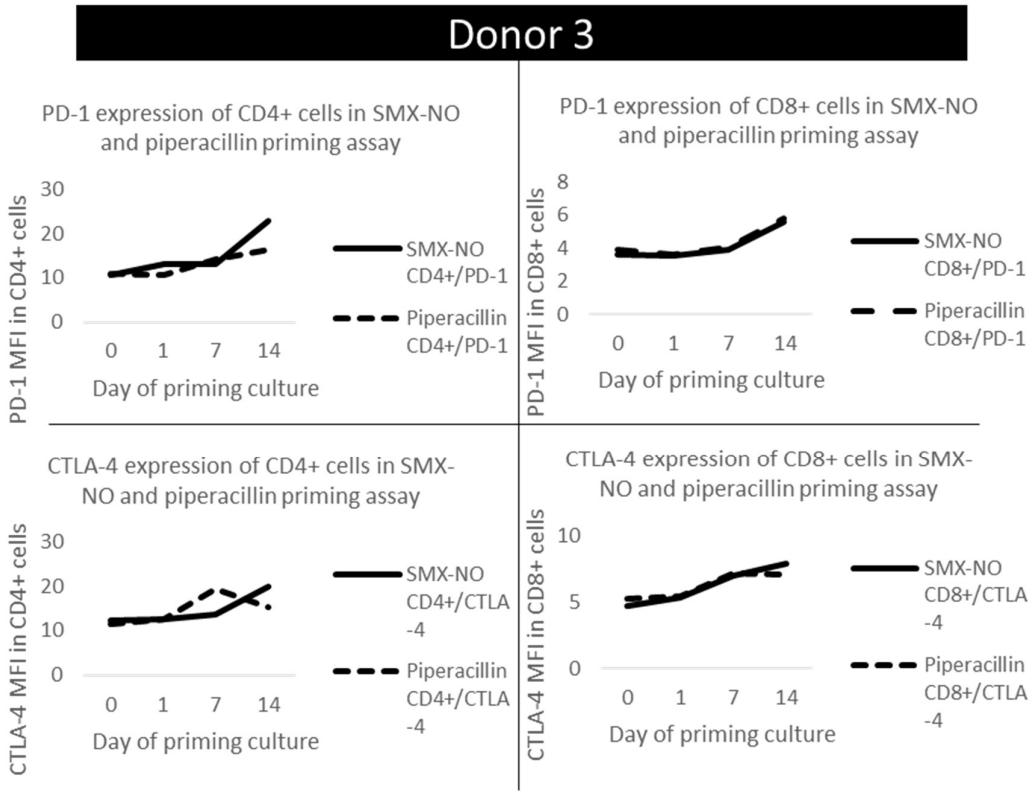


Figure 4.3 T cell expression of PD-1/CTLA-4 in response to drug treatment - *Naïve T cells ($2.5x10^6$ / well) obtained from healthy volunteers were co-cultured with autologous moDC ($8x10^4$ / well) with either SMX-NO (40μM) or piperacillin (2mM) for 14 days in a 24-well plate. T cells were then harvested on days 0, 1, 7 and 14 and stained with cell surface marker antibodies against CD4, CD8, and PD-1/CTLA-4. Cells were analysed by flow cytometry on a BD FACS CANTO II. Gating strategy can be seen above.*

MFI of PD-1/CTLA-4 expression in CD4/CD8 T cells was determined at each of the four time points and displayed in the graphs above to determine overall CD4/CD8 T cell population expression of the co-inhibitory checkpoint markers during the course of naïve T cell priming.


Donor 2
PD-1 expression of CD4+ cells in SMX-NO and piperacillin priming assay
PD-1 MFI in CD4+ cells
40
30
20
10
0
0 1 7 14
Day of priming culture
SMX-NO CD4+/PD-1
Piperacillin CD4+/PD-1
PD-1 expression of CD8+ cells in SMX-NO and piperacillin priming assay
PD-1 MFI in CD8+ cells
8
6
4
2
0
0 1 7 14
Day of priming culture
SMX-NO CD8+/PD-1
Piperacillin CD8+/PD-1
CTLA-4 expression of CD4+ cells in SMX-NO and piperacillin priming assay
CTLA-4 MFI in CD4+ cells
30
20
10
0
0 1 7 14
Day of priming culture
SMX-NO CD4+/CTLA-4
Piperacillin CD4+/CTLA-4
CTLA-4 expression of CD8+ cells in SMX-NO and piperacillin priming assay
CTLA-4 MFI in CD8+ cells
15
10
5
0
0 1 7 14
Day of priming culture
SMX-NO CD8+/CTLA-4
Piperacillin CD8+/CTLA 4


Donor 3
PD-1 expression of CD4+ cells in SMX-NO and piperacillin priming assay
PD-1 MFI in CD4+ cells
30
20
10
0
0 1 7 14
Day of priming culture
SMX-NO CD4+/PD-1
Piperacillin CD4+/PD-1
PD-1 expression of CD8+ cells in SMX-NO and piperacillin priming assay
PD-1 MFI in CD8+ cells
8
6
4
2
0
0 1 7 14
Day of priming culture
SMX-NO CD8+/PD-1
Piperacillin CD8+/PD-1
CTLA-4 expression of CD4+ cells in SMX-NO and piperacillin priming assay
CTLA-4 MFI in CD4+ cells
30
20
10
0
0 1 7 14
Day of priming culture
SMX-NO CD4+/CTLA-4
Piperacillin CD4+/CTLA-4
CTLA-4 expression of CD8+ cells in SMX-NO and piperacillin priming assay
CTLA-4 MFI in CD8+ cells
10
5
0
0 1 7 14
Day of priming culture
SMX-NO CD8+/CTLA-4
Piperacillin CD8+/CTLA-4

4.4.4 Assessment of the effects of blocking PD-1 and CTLA-4 in the naïve T cell multi-well priming assay (T-MWA)

After analysing the fluctuations in expression of PD-1 and CTLA-4 in response to different drug treatments, we investigated the direct effect of co-signalling on drug-specific T cell priming with proliferation as a readout. For this, the T-MWA was performed on different healthy donors as in chapter 3, but instead used blocking antibodies to abrogate co-signalling and its effects on priming. This meant proliferative responses to: drug alone, drug + PD-L1 block (used to block PD-1 ligand on APCs and abrogate effects of PD-1), and drug + CTLA-4 block.

It must be stated that whilst overall success of priming was determined based on statistical evaluation (significant – $p<0.05$ – increase in proliferation) of many individual wells treated under identical conditions, positive priming can be observed sporadically in some individual wells. Therefore, this could also be taken into consideration (albeit lesser than overall statistical significance of T-MWA) when evaluating the potential immunogenicity of a drug.

Figure 4.5 shows the first experiment in which T-MWA was performed with SMX-NO and piperacillin treatment. For SMX-NO treatment; significant proliferation responses ($p<0.05$) were observed with SMX-NO alone, and with either block applied. A larger increase in proliferation was observed compared to its own control in the presence of the co-inhibitory blocks, but there was no significant increase when compared directly against the proliferative readouts of drug alone. Piperacillin priming failed across the board for this donor; however, 3 individual wells displayed an increased proliferative response when re-stimulated with piperacillin (2 with PD-L1 block, and one with CTLA-4 block).

Figure 4.6 again shows positive priming to SMX-NO with significant increases in proliferation observed in drug alone, and each block. However, this time, there was a significant increase in proliferation when comparing drug alone, to drug + CTLA-4 block, which induced the greatest proliferative response. Piperacillin priming failed to drug alone, whereas there were significant increases in proliferation upon addition of either of the co-inhibitory blocks (with PD-L1 block eliciting the greatest effect on proliferation. Priming to flucloxacillin and DDS-NO failed across the board, irrespective of the use of co-inhibitory block.

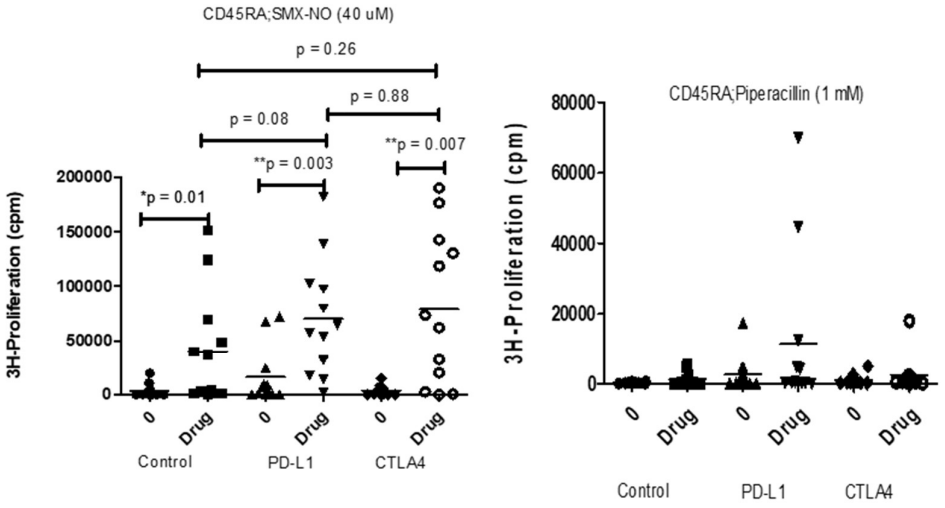


Figure 4.5 *Effects of blocking co-inhibitory pathways PD-1/PD-L1 and CTLA-4 on antigen specific T cell proliferation in response to drug treatment with four different drugs. Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40μM) or piperacillin (1mM); both with and without the presence of blocking antibodies against PD-L1 and CTLA-4. After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40μM), piperacillin (1mM) or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Data was plotted as scatter plot; Mann-Whitney test performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug treatment. Statistical significance was determined as; *$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$.*

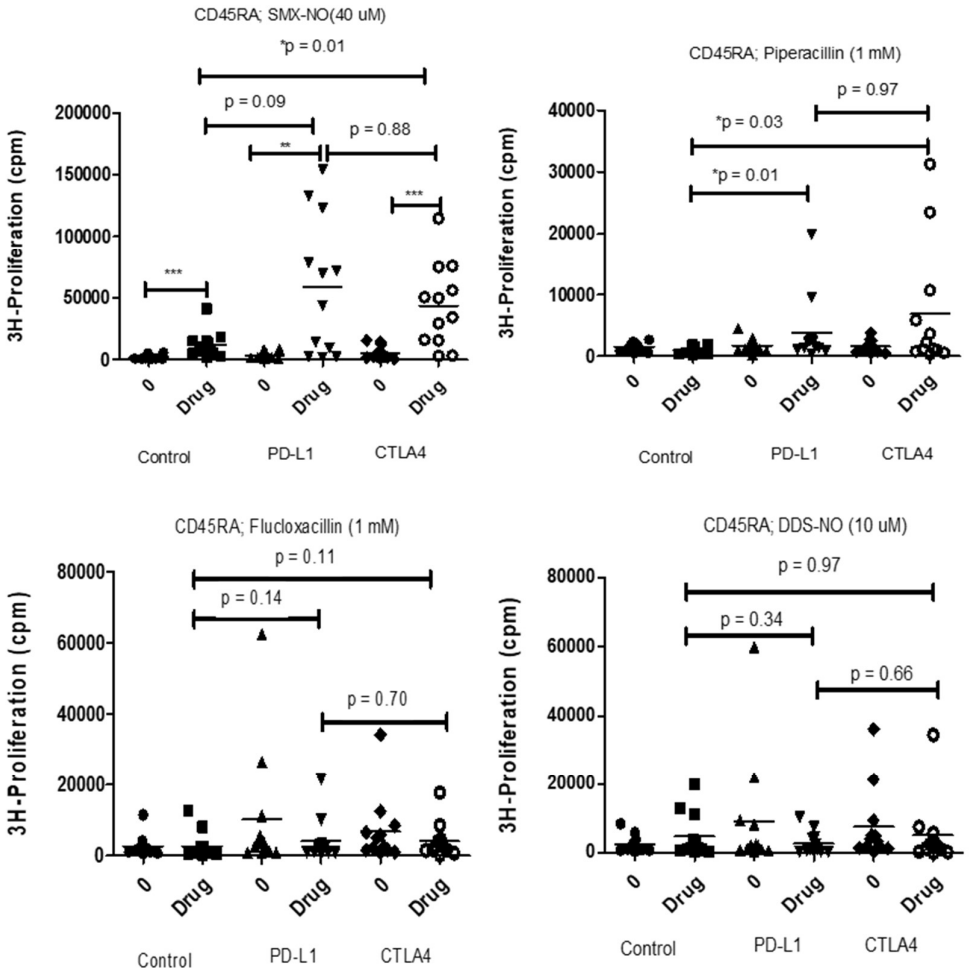


Figure 4.6 *Effects of blocking co-inhibitory pathways PD-1/PD-L1 and CTLA-4 on antigen specific T cell proliferation in response to drug treatment with four different drugs. Naïve T cells ($2x10^5$ / well) were plated in a 96-well plate with autologous moDC (8000 / well) with SMX-NO (40μM), Flucloxacillin (1mM), piperacillin (1mM) or DDS-NO (10uM); both with and without the presence of blocking antibodies against PD-L1 and CTLA-4. After 14 days plates were centrifuged, and cells were delicately washed to dispose of free drug in the media. Cells were gently re-suspended and re-challenged with SMX-NO (40μM), Flucloxacillin (1mM), piperacillin (1mM), DDS-NO (10uM) or media as a negative control; 8 wells of negative control, 40 drug-treated wells. After 48-hour incubation 3H-thymidine (0.5μCi / well) was added for 16 hours before plates were harvested and proliferation counted on a beta-counter. Data was plotted as scatter plot; Mann-Whitney test performed to determine statistical significance of T cell proliferation of drug-treated wells compared to no drug treatment. Statistical significance was determined as; *$p \leq 0.05$; **$p \leq 0.005$; ***$p < 0.001$.*

4.5 DISCUSSION

The advent of numerous *in vitro* diagnostic and predictive test systems such as the LTT and naïve T cell priming assay have furthered our understanding of DHR. However, these current systems are not perfect, and are not yet fully fit for purpose. For diagnostic testing the LTT remains one of the more popular assays; whilst it is easy to perform and has become increasingly useful in patient cohorts where *in vivo* testing is more difficult, there is still a problem with sensitivity (~50%) despite its high specificity (upto 95%) [273]. Prediction of DHR and the potential immunogenicity of a drug or compound is what is truly needed in the field. Indeed, the naïve T cell priming assay as well are T cell cloning are used extensively by our lab with this question in mind. Whilst these assays are currently the best on offer, and can be used to study mechanisms when a problem has been identified by the clinic, there are a few questions that need to be answered. For example, we still currently struggle to prime naïve T cells to drugs with MHC class II restriction; whilst we regularly prime to those with MHC class I restriction. Additionally, we sometimes struggle to prime to a parent drug, and instead must prime to its reactive metabolite (i.e. SMX-NO instead of SMX).

HLA risk alleles have been implicated in the onset of DHR with specifically interacting drugs; as certain proteins encoded by HLA alleles are able to interact specifically with a drug derived antigen, causing immune response and subsequent DHR. Perhaps the best example of this is HLA-B*57:01 association with Abacavir induced hypersensitivity which has been demonstrated to be the only association robust enough to be implemented widespread in the clinic; whilst other associations such as HLA-B*57:01 have been demonstrated through GWAS and experimental studies, but have not yet been implemented clinically as they are not as consistently trustworthy [274]. Indeed, the majority of people who possess a supposed HLA risk allele

do not actually develop DHR when exposed to the drug/antigen, therefore, there must be some other factors to be considered when trying to predict whether a DHR will occur.

In addition to this, in the previous chapter we optimised the traditional naïve T cell priming assay [71] to develop a two new assays: T-MWA and T-MDA. The T-MWA allows us to more closely analyse the immune response and naïve T cell priming and takes into consideration the precursor frequency of T cells necessary to reach the activation threshold. This allows for analysis of the strength of the induced response as well as the number of wells stimulated by a drug. As the T-MWA still consists of only moDCs, naïve T cells, and drug we do not know if we are fully replicating the optimal immune microenvironment for priming T cells. The assay does not consider the full repertoire of immune regulation that participates in immune activation and responses; as immune activation is dependent not only on signal 1 (the formation of a APC-TCR immune synapse), but is heavily reliant on the balance of signal 2 axis of co-stimulatory and inhibitory factors, such as PD-1 and CTLA-4, in addition to the action of other immunosuppressive cell types such as Tregs.

In this chapter, the presence of, and importance of signal 2 (co-inhibitory factors and Tregs) during naïve T cell priming was investigated in response to the use of different drugs/antigens. We also investigated the functional outcome of blockade of the co-inhibitory factors PD-1 and CTLA-4. This information can help us further understand the immunological pathomechanisms of DHR in response to different drug treatments; explaining why different drugs have different effects, and may also help elucidate why HLA risk alleles are not always an accurate measure of predisposition to DHR upon specific antigen exposure. This additional information may also help us further

optimise our *in vitro* predictive assay conditions; providing a more comprehensive, accurate representation of the considered immune microenvironment and signal 2.

In our Treg expression analyses we compared the presence of Tregs, proliferating from the naïve population at days 0, 1, 7, and 14 of our priming cultures, in response to treatment with either SMX-NO or piperacillin. Across all three donors there was an overall increase in Tregs from day 0-14 with both drugs used; similar responses have been described in response to anti-convulsant drug therapy, whereby cytotoxic T cells have been shown to switch to Tregs during DHR immune responses [275]. Furthermore, the ratio of FOXP3 Tregs increases in patients with DHR/DRESS [276]. These findings indicate that the immune system attempts to protect the host at very early stages of an effector T cell response. This also confirms the transient changes of T cell phenotype from CD4 to CD8 in two of three donors, and the vast increase in CD4:CD8 ratio observed in the first donor in *figure 4.2* as the T cells undergo priming they can fluctuate between a state of CD8 cytotoxicity, and Treg/CD4+ memory cells. Other studies have shown similar expansion of Tregs in acute stages of DHR such as TEN; coupled with massively impaired functionality of the immunosuppressive cell type which can help to explain the immune systems inability to regulate immune responses in such severe circumstances [277]. Now that we have shown there to be an increase in Tregs during T cell priming, it would be important to determine their functional effects in the constructs of our *in vitro* screening systems. Of particular importance would be the assessment of whether these Tregs are drug responsive, and if so, what is their method of immune-suppression. Following priming, we attempted to remove the generated Tregs using MACS CD25+ beads and cell separation columns, before re-stimulating cells with drug to compare with 'standard' priming culture. Unfortunately, in preliminary attempts this yielded a complete failure of the assay – presumably as

the harsher treatment (37 down to 4 degrees Celsius, then back to 37 during the separations), washes and passage through the cell separation column caused a loss of function in the remaining T cell populations. Interestingly *Gibson et al.,* discovered that introduction of autologous Tregs into a priming culture can significantly dampen antigen specific T cell priming [182]. If more time were available to us, we would have spent time to optimise this experiment, as the implication of Tregs in DHR are clear, particularly when considering the connection to other signal 2 components such as CTLA-4 which we will move on to next.

Upon analysis of the mean expression of co-inhibitory markers PD-1 and CTLA-4 upon drug treatment during the course of our priming cultures, we observed fluctuations in expression of each of the two co-inhibitory regulators. This worked aimed to determine any observable differences in PD-1 and CTLA-4 expression upon different drug treatments. Across the three donors there were transient increases in PD-1 expression in the CD4+ T cell populations, which was mirrored by CTLA-4 expression. The same trend can be observed under the same conditions when considering only the CD8+ T cell subset in 2 out of 3 donors; whilst in the third donor there were marginal decreases observed in both PD-1 and CTLA-4 expression to each of the two drug treatments. Interestingly, there was a decrease in expression observed between days 7 and 14 in the SMX-NO treated CD4+ T cells in 2 out of 3 donors, and in the piperacillin CD4+ treated T cells in 1 out of 3 patients; mirroring the decreased Treg abundance between days 7 and 14 from our previous work. This fluctuation in expression of the co-inhibitory markers may serve to highlight its potential importance of these two receptors in immune activation and downstream DHR. On balance, SMX-NO induces a greater expression of PD-1, whereas piperacillin induces a greater

increase in CTLA-4. However, the observed differences between drug treatments are marginal.

There is an overall increase of the both PD-1 and CTLA-4 during our priming cultures, perhaps increased to maintain tolerance during the priming of naïve T cells. Similar studies have also observed such increases, for example, antigen specific T cells isolated from multi-drug hypersensitivity patients had augmented PD-1 expression [278]. Previous studies within the group have described similarly increased expression profiles of PD-1, CTLA-4 and another co-inhibitory contributor TIM3 [182]. Now that we had observed increases in expression profiles of PD-1 and CTLA-4 in our priming cultures, it was important to then highlight whether this is translated functionally in our T-MWA; as PD-1 and CTLA-4 expression increases during priming, does their blockade translate to a functional decrease in antigen-specific T cell priming and proliferation? The literature has certainly observed a greater increase in risk of allergy when PD-1 expression is low [279], whilst there is a hugely increased risk of adverse events when PD-1 and CTLA-4 are blocked in cancer immunotherapy treatments [280,281]. This gives credence to the idea that blockade of PD-1 and CTLA-4 should be able to increase the degree of proliferation observed in our T-MWA, as achieved in the previous traditional priming assays [137,182], and may even be able to restore positive naïve T cell priming in instances of failed priming to certain drugs.

An increase in the intensity of proliferation amongst the SMX-NO treated wells was observed with all donors tested using the T-MWA to look at the effects of PD-1 and CTLA-4 blockade. The individual number of wells that responded to SMX-NO on the scatter plot, and in terms of statistical significance of overall proliferation were both increased when compared to its own control. It must be stated that not only does the intensity of the drug treated wells increase upon co-inhibitory blockade, but the control

well proliferative intensity also increases upon use of PD-L1/CTLA-4 block. This could be explained due to the lack of regulation after co-inhibitory block, leading to decreased activation threshold of T cells that can now respond to nascent stimuli in the wells, causing increased basal proliferation. In all tested donors, piperacillin, flucloxacillin and DDS-NO treatment yielded statistically non-significant priming, despite individual wells in the scatter plot showing increased proliferative intensity. The increase in background influenced by co-inhibitory blockade meant that no statistical significance in overall proliferation was observed. However, it was noted that there was statistically significant increase in proliferation observed when comparing drug treated wells to drug + block treated wells, opposed to their own control in piperacillin treated wells.

Although blocking the co-inhibitory pathways increased the intensity of the immune response, negative priming in wells treated with drug alone was generally not reversed upon blockade of the checkpoints - as observed previously using the standard priming assay [137,182]. This is perhaps unsurprising given that treatments using immune checkpoint inhibitors can cause lowered immune activation threshold which can lead to severe adverse events [174,178,221,250]. Whilst we weren't able to achieve a reversal from negative to positive priming in instances where priming had failed with drug alone, we did see increases in proliferation in individual wells. Although antigen-specific proliferation was not significant in some instances, there was an overall increase in T cell proliferation upon checkpoint inhibition, presumably due to the decreased activation threshold leading to non-antigen specific T cell proliferation that could still participate in an immune attack in DHR *in vivo.*

If additional time were available, we would have liked to perform mechanistic evaluation of the depletion of Tregs by removing the generated population post-

priming to investigate the difference they make to the readout proliferative response. Co-signalling is a vast network of co-inhibitory and co-stimulatory factors. Whilst other co-inhibitory checkpoints such as TIM-3 have been shown to be of lesser importance than PD-1 and CTLA-4 [137,182], it would be interesting to investigate the role that co-stimulation can play in our assays such as CD28 that competes for its ligands with CTLA-4 and has been therapeutically modulated to prevent immune eruptions and autoimmunity through organ rejection following transplantation [282].

We achieved the aims set out in this chapter as we were able to ascertain the involvement of immune regulation in our *in vitro* assays: Tregs were generated from the naïve T cell populations during priming, there were increases in expression of both PD-1 and CTLA-4 during the course of priming, and blockade of the co-inhibitory checkpoints led to an increased lymphoproliferative response. This clearly shows the importance of immune regulation in DHR and in our assays. Further investigation must take place to understand the importance of immune regulation to the fullest extent. We can then use this information to further improve the T-MWA in particular and may be able to use this knowledge to improve patient outcomes in diagnostic assays for DHR.

5 APPLICATION OF THE LYMPHOCYTE TRANSFORMATION TEST FOR USE AS A CAUSALITY TOOL FOR THE DIAGNOSIS OF PAEDIATRIC DHR.

5.1 INTRODUCTION

Drug hypersensitivity reactions are becoming an increasing issue in the medical field, causing high morbidity iatrogenic illness that costs the healthcare system millions of pounds per year. This in main is due to the filling of hospital beds by this potentially preventable illness, the cessation of treatment of otherwise useful drugs causing wastage, and the removal of useful drugs from market, in addition to the treatment of patients suffering from DHR with alleviating drugs.

One group of patients that are at an increased risk to DHR are patients being treated for cystic fibrosis (CF). This life limiting autosomal recessive disease affects in the region of 70,000 people worldwide; mainly Caucasians. If left untreated prognosis can be expected to be less than ten years, whilst current treatment strategies have led to the median age of mortality in CF sufferers to rise to 37 years old. The majority of deaths (>80%) associated with the disease are due to airway infection and longer term obstructive disease of the lungs [283]. The disorder is caused by dysfunction of the chloride channels of the endocrine glands, specifically, the cystic fibrosis transmembrane conductance regulator (CFTR). This occurs due to gene mutation and affects multiple internal organs; mainly the lungs and respiratory systems. But can also negatively affect the pancreas, liver and respiratory organs to varying degrees [284]. The discovery of the CFTR gene 30 years ago [285] has vastly increased the scope of prospective therapies available to sufferers, as research into the development of personalised gene therapies have become increasingly popular. These include both viral and non-viral gene therapies, as well specific molecule therapies aimed one of the 1900 identified CFTR causative mutations [286]. The addition of a fully-functioning CFTR gene into defective airway cells has also been put forward as the purported best way to treat CF for pan-CFTR mutations [287]. Despite these genetic advancements in

an effort to cure CF, the main mechanism of improving the prognosis for those diagnosed with the disease, is to target end-point airway destruction through the administration of single or combination therapy of antibiotics against bacteria such as *pseudomonas aeruginosa* [288]. This has led to this particular group of patients becoming particularly reliant on long-term treatment regimens of parenterally administered antibiotics; penicillin(s), cephalosporins, and aminoglycosides, and as such, have increased risk of DHR when compared to other patient groups [289]. Whilst relatively low morbidity skin-based reactions such as rash and pruritis are the most common, these patients can also suffer more severe skin reactions, causing high morbidity, and in even more extreme cases, mortality. In some instances of antibiotic induced DHR there are immediate IgE mediated anaphylaxis, however, the majority are late-onset type IV DHRs that manifest in the aforementioned skin rash as well as fever [290]. The increased lifespan of CF patients due to advances in antibiotic therapy has become a double-edged sword, as the longevity of this treatment plan can be brought into question as DHR increases due to the long-term nature of high dose antibiotic administration. These reactions are not currently predictable based on the pharmacology of the drug, and are especially difficult to diagnose, owing to the fact that there is no current global consensus on whether single antibiotic or combination antibiotic administration is the most effective treatment plan [288]. It is imperative to be able to accurately 'look through the noise' of these multiple drug regimens and diagnose actual DHR; with the correct culprit drug elucidated so that there is not cessation of treatment for other correctly functioning beneficial drugs. Despite long-term drug administration being a major cause of DHR, paediatric CF patients are also at risk of adverse events and can be even more difficult to diagnose due to the invasive nature of current diagnostic tools that are not fit for purpose.

Misdiagnosis of DHR is a common problem in children, and can lead to cessation of treatment, causing risk to the child as treatment options become increasingly limited and the risk of infection heightens. This is evidenced by the fact that despite 10% of parents reporting suspected drug allergies in their children, only a few are positively confirmed following current diagnostic evaluation [291]. The use of other drugs due to misdiagnosis also leads to increase antibiotic resistance and increased costs for the healthcare system [292]. Misdiagnosis and under-diagnosis are both a major issue, as misdiagnosis can prevent the child from receiving potentially life-lengthening treatment, whilst under diagnosis can cause to increased risk of high-morbidity or mortality DHR. In order to properly manage drug allergy in children, it is important to be able to accurately diagnose true DHR [293], this is especially true of paediatric CF patients where the culprit drug must be identified in multiple-drug therapy to maintain effective treatment of airway disease. Of course, as the number of drugs involved in the treatment increase, the likelihood of the current diagnostic tests being able to accurately detect the true culprit drug decreases.

Current *in vivo* diagnostic techniques to identify drug allergy have already been discussed in Chapter 3. It can be said that the issues elucidated for tests such as the skin prick, skin patch, and drug challenge tests, can be amplified in this instance as *in vivo* tests in children can be complicated and difficult to use effectively due to their invasive and risky nature in patients suffering severe cutaneous adverse reactions (SCARs) in particular. There is a need for a non-invasive, specific and sensitive test for this group of vulnerable patients, as the importance of correct diagnosis of DHR in paediatric patients is of even greater importance than that of adult patients when attempting to improve prognosis through long-term administration of antibiotics. This treatment is a ‘necessary evil’ until an effective, safe, gene therapy is developed, which

can cure CF through reinstation of a functioning CFTR gene in the airway cells of those suffering from the disorder. It is for this reason that this chapter will focus mainly on the use of the lymphocyte transformation test (LTT) in the diagnosis of drug allergy in patient samples. The utility of the assay will be discussed which will include the positive attributes of the assay, as well as the drawbacks. Whether or not the assay is fit for purpose in this instance will also be decided, and a list of potential improvements to the assay will also be put forward.

For the LTT to be correctly discussed, it must first be recognised that it is one of multiple *in vitro* tests developed with the intention of accurate DHR diagnosis – others including the CD69 leukocyte activation test, and the ELISpot assay (which our lab in particular uses to characterise expression and cytokine secretion from different T cell subsets including clones.

Upregulation of lymphocyte activation marker CD69 can be detected by flow cytometry in order to determine activation of T cells in response to drug treatment within 48 hours. Studies have shown that when compared to the LTT which takes 6 days to complete, 15 out of 15 positive LTT samples also showed CD69 upregulation within two days. It was argued that this can be a preferable alternative to the LTT in situations of severe DHR such as SJS/TEN where rapid diagnosis is vitally important [242]. Other studies have compared CD69 upregulation to *in vivo* patch tests, concluding that four out of six patients that were skin patch positive, were also positive for CD69 upregulation [294], which although limited, does not show this method to be equivalent to the more established *in vivo* tests. That said, CD69 upregulation remains an important consideration in drug allergy, especially owing to its involvement in immune regulation through control of Treg differentiation and secretion of IFN-γ, IL-17 and IL-22 [295]. The detection of secreted cytokines such as IFN-γ is important for

the profiling of T cell responses and has been used regularly to diagnose cutaneous DHR. ELISA, electrochemiluminescence and flow cytometry can all be used to achieve this, but the ELISpot in particular has been used by groups such as ours to great effect over the years and is a mainstay of immune profiling of T cells [50,225,296,297]. Whilst its use in immune profiling has been useful, some studies have shown that the sensitivity of the ELISpot is 100%, but only achieved sensitivity of 47%, lower than that of the ELISA and flow cytometry (50% and 57% respectively) [298]. Another issue with current *in vitro* testing is the inability to conclude that one of the tests is suited to diagnosing all types of observed manifestations of DHR. There is currently no comprehensive test for DHR diagnosis as the utility of each of the *in vitro* methods depend on the mechanism involved [299,300]. One of the main concerns surrounding *in vitro* tests is that whilst generally displaying excellent specificity (approx. 100%); the sensitivity of these tests leaves much to be desired, and so the risk of false positives is relatively high. This is particularly problematic for this vulnerable population of paediatric CF patients as misdiagnosis and drug withdrawal is one of the major concerns surrounding their treatment plan and is already the main reason for the need for better diagnostic testing. A major theme concerning *in vitro* testing is that there is an inherent need to develop one particular improved diagnostic test for DHR that can work in all situations, spanning different treatment strategies, drugs used, and immune mechanisms involved [301].

The LTT has been used to test for drug allergy for over forty years – *Warrington et al.,* utilised the LTT to a suspected DHR causing drug in 1979; the assay was accurate in diagnosing immediate reaction, but only detected positive responses in 3 out of 11 patients with delayed-type reactions to penicillin. Nine out of eleven patients showed positive LTTs for isoniazid induced DHR, whereas only eight out of thirty-one

displayed positive LTT responses when the suspected drug was penicillin [302]. PBMC are isolated from blood donation and then cultured for 6 days with the suspected culprit drug before proliferation is measured through addition of tritiated thymidine and counting on beta-counter. Proliferation of drug challenged cells is compared to negative control cells, with stimulation index being calculated by CPM stimulated cells/CPM medium only treated cells; tetanus or PHA can also be used as a positive control to ensure cells have the propensity to proliferate. Further studies confirmed the application of the LTT in detecting T cell responses in patients presenting with liver dysfunction attributed to isoniazid, once again with a high specificity (83-90%) but only 50% sensitivity [303]. Due to its versatility in being able to diagnose DHR in different classes of antibiotics, the LTT is deemed superior to the competing *in vitro* tests, as the need for a single solution in becoming increasingly important. Additionally, despite the sensitivity of the assay being relatively low (60-70%) for detection of DHR to beta-lactam antibiotics, this is still higher than competing assays [26].

Clearly, there is an inherent need for a one size fits all *in vitro* diagnostic test to specifically, and sensitively detect the culprit drug responsible for DHR in a patient. The test must work well across a range of drugs with different immune mechanisms at play. It can be argued that currently, the closest assay available to fit the above criteria is the LTT. Despite this, there are issues with the current version of the assay such as: number of PBMC required makes it difficult to test a range of drugs and concentrations in paediatric samples, the assay has relatively low sensitivity causing false negatives and drug withdrawal which is another major issue for paediatric CF patients, and cross-reactivity between different antibiotics/classes of antibiotic coupled with low sensitivity makes it difficult to discern the culprit drug in multi-drug

treatment strategies. This chapter will utilise the LTT to diagnose DHR, detecting the culprit drug in multiple-drug therapy in paediatric patients with CF. These data will help to assess whether the current assay is fit for purpose. The main aim is to elucidate both the strengths and weaknesses of the current assay, highlighting areas in which we can improve this for both adult and paediatric patients.

5.2 CHAPTER AIMS

1. Assess the utility of the LTT as a tool for the diagnosis of DHR in paediatric patients undergoing multiple antibiotic treatment from different classes.
2. Establish whether or not the LTT is for purpose.
3. Formulate new ideas how we can improve *in vitro* diagnostic testing for DHR – especially for vulnerable patients such as CF paediatrics.

5.3 METHODS

All methods for techniques seen in this chapter can be found in detail in Chapter 2: Methods and Materials.

5.4 RESULTS

During the course of this chapter the utility of the LTT for the diagnosis of DHR in paediatric CF patients' blood samples is assessed. All samples analysed by LTT in this chapter were received through ongoing collaborations with the Royal Manchester Children's Hospital, and St James' Hospital in Leeds cystic fibrosis wards. Informed consent was obtained from all parents for the blood donations. Due to the extremely sensitive nature of paediatric patient data, the only information provided alongside the samples was: anonymised patient code, antibiotics currently being used in treatment, the fact that they had a suspected DHR (generally skin reaction), and the fact that they were under 16 years of age. As *in vivo* diagnostic tests were not suitable for use in

patients of this age, we were providing a service to attempt to diagnose the culprit causative drug through use of the LTT; this was not confirmed through *in vivo* tests such as skin patch or drug challenge. All PBMC were isolation on arrival of the blood sample, and all LTTs were performed on freshly isolated PBMC (not frozen) for consistency and to maintain the integrity of the immune cells for the function of the assay. The proliferative propensity of the PBMC were confirmed through stimulation with either tetanus toxoid (TT) or the mitogen phytohaemaglutinnin (PHA).

5.4.1 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH BOTH FLUCLOXACILLIN AND BENZYLPENICILLIN

As can be seen in *figure 5.1* the first sample received (LANC001) had a suspected DHR during the course of treatment with the beta-lactam antibiotic flucloxacillin and benzylpenicillin (penicillin G). Proliferation was observed only when PBMC had been stimulated with PHA, however, treatment to a range of concentrations of both flucloxacillin and benzylpenicillin did not cause significant increase in proliferation when compared to base level negative control (no drug).

5.4.2 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH BOTH FLUCLOXACILLIN AND RIFAMPICIN

Figure 5.2 displays the data achieved from the second patient sample received. The patient was again being treated with flucloxacillin, but instead of benzylpenicillin was receiving rifampicin when they suffered a suspected adverse event. Again, proliferative viability of the cells was displayed after stimulation with PHA, but no significant increase in proliferation was observed in response to flucloxacillin treatment at any concentration tested when compared to negative control. However, rifampicin treatment did result in a significant increase in proliferation ($p<0.05$) at a concentration of 12.5µM.

5.4.3 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH FLUCLOXACILLIN OR MEROPENEM

The next two patient samples received (MAN001 & MAN003) are displayed in *figure 5.3* as each of them were only receiving treatment of a single antibiotic at the time of suspected DHR occurrence. Note: MAN002 sample was destroyed due to incorrect tube being used when sample was collected resulting in coagulation of the blood during transit. MAN001 was being treated with flucloxacillin when suspected DHR was observed. Marginal increase in proliferation compared to baseline was seen across multiple concentrations of flucloxacillin testing; with significant increase ($p<0.05$) observed upon treatment with 0.5mM flucloxacillin. MAN003 was being treated with meropenem, but no increase in proliferation was observed across any of the range of concentrations tested (0.25-4mM). The proliferative nature of the cells was confirmed through stimulation with TT to ensure lack of proliferation upon drug treatment was not due to natively unresponsive or damaged cells.

5.4.4 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH BOTH FLUCLOXACILLIN AND MEROPENEM

MAN004 seen in *figure 5.4* was being treated with both flucloxacillin and meropenem. In this instance, we were not able to detect the suspected culprit drug. Despite being tested at a range of concentrations (0.25-4mM) a significant immune response in the patients' PBMC was not detected.

5.4.5 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH: TEMICILLIN, TRIMETHOPRIM, CIPROFLOXACIN, TEICOPLANIN, TAZOCIN AND MEROPENEM

Next a sample was received (MAN005 – *figure 5.5*) where the patient had been receiving multi-drug antibiotic therapy for CF induced airway infection to six different

antibiotics: temicillin, trimethoprim, ciprofloxacin, teicoplanin, tazocin and meropenem. In this instance LTTs were performed to all six drugs to a range of concentrations (5 different concentrations to tazocin and meropenem, 4 different concentrations to each of the other drugs), with a view to elucidating the culprit drug(s) that induced the suspected DHR in the patient. Significant proliferative responses were observed with the following drugs: temicillin (25 and 50µg/mL), trimethoprim (12.5µg/mL), tazocin (1, 2 and 4mM) and meropenem (4mM). No significant proliferative responses were observed for ciprofloxacin and teicoplanin, whilst TT successfully stimulated cells in all instances.

5.4.6 LTTs ON PAEDIATRIC PATIENT PBMC TREATED WITH EITHER MEROPENEM AND CEFTRIAXONE OR TAZOCIN, AMPHOTERICIN B AND VANCOMYCIN

The culprit drug(s) responsible for the suspected DHR were not outlined by the LTT in either of the next two patients (MAN006 – *figure 5.6*, MAN007 – *figure 5.7*). MAN006 was being treated with meropenem and ceftriaxone and was negative across the range of concentrations tested, whereas MAN007 was tested for immune response against tazocin, amphotericin B and vancomycin in multiple concentrations; no significant responses were observed to any of the drugs at any concentration. Again, proper immune function was observed through PHA stimulation.

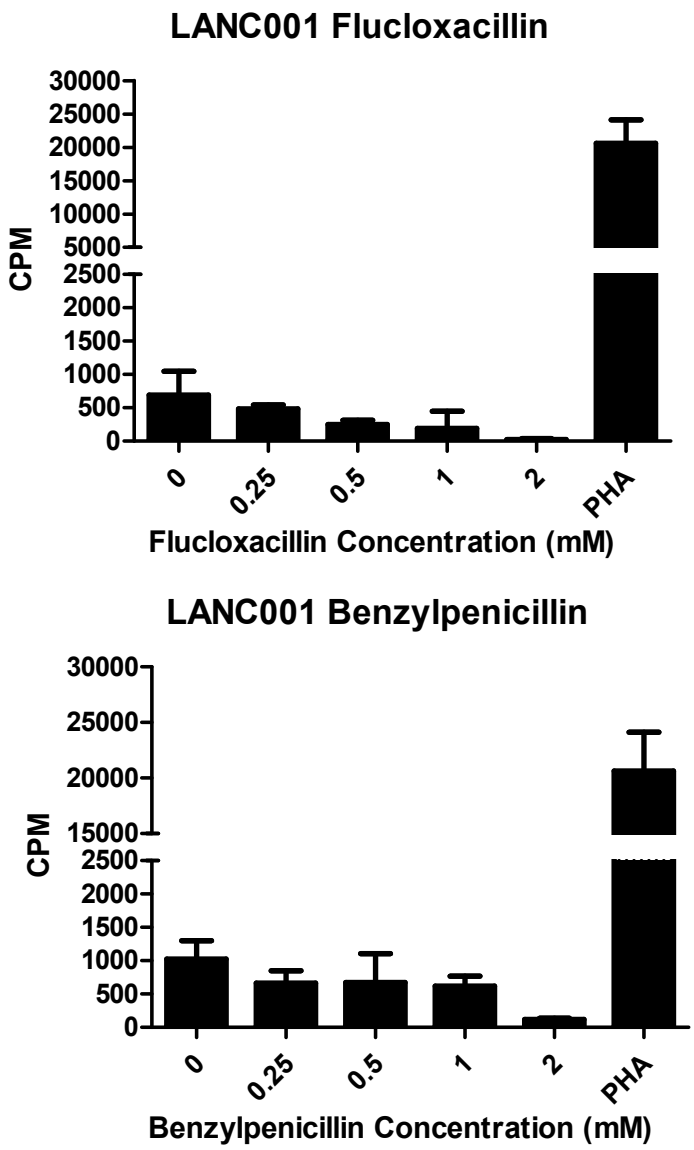


Figure 5.1 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5μCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

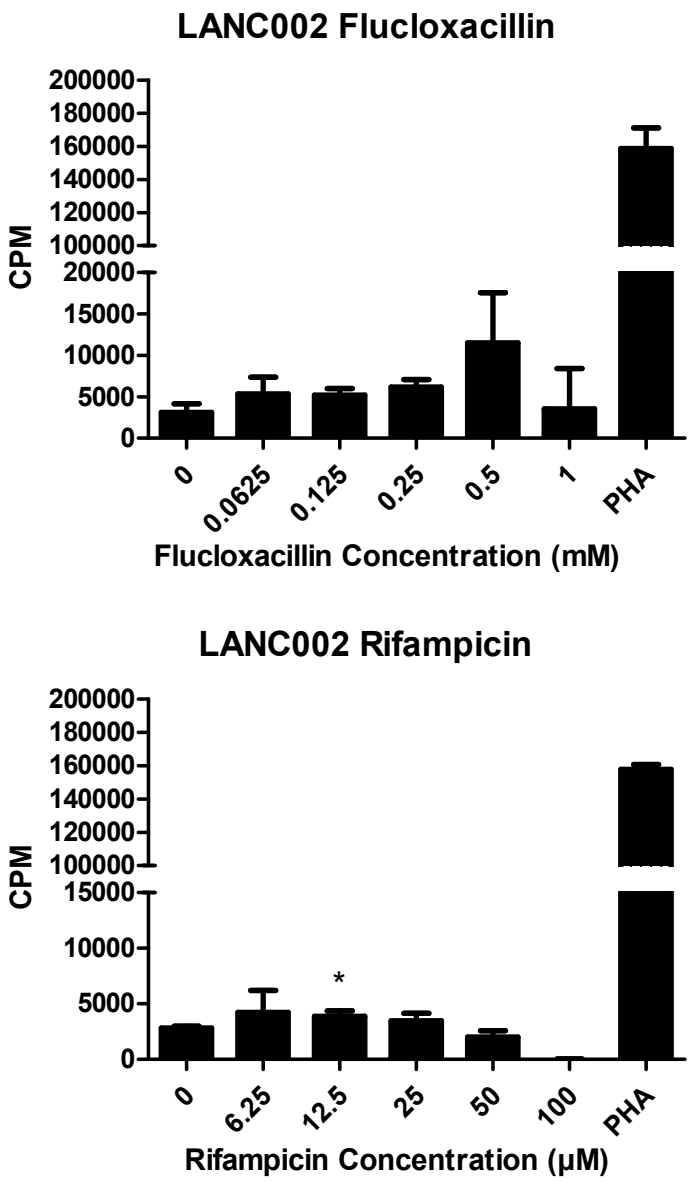


Figure 5.2 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5µCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

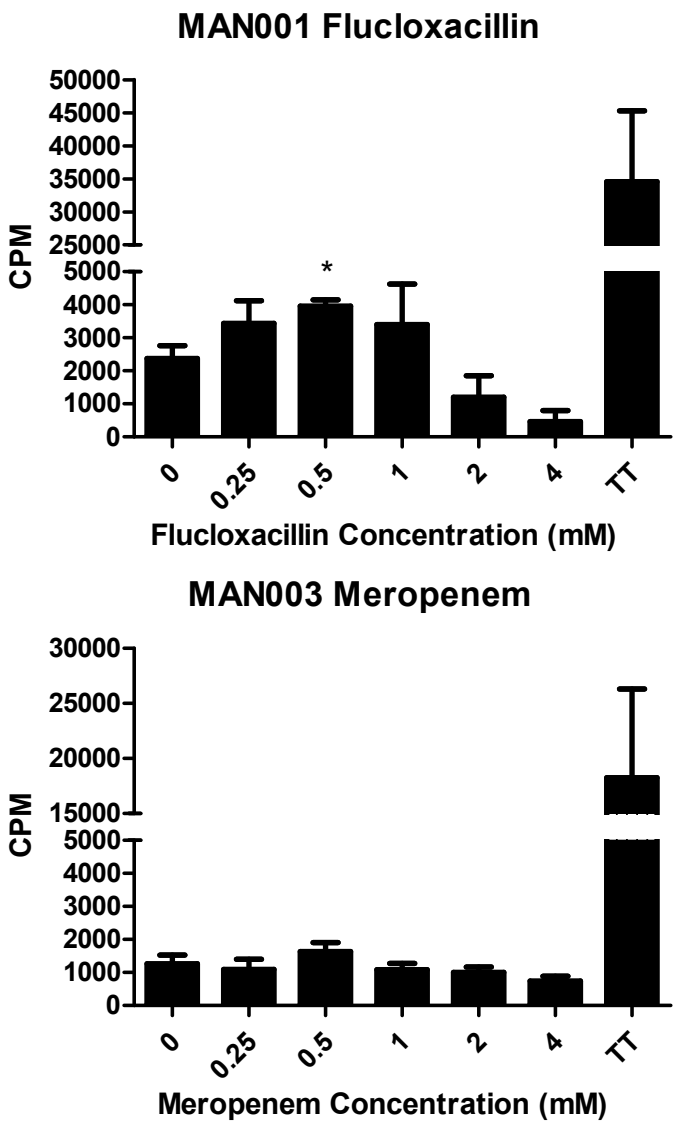


Figure 5.3 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5μCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

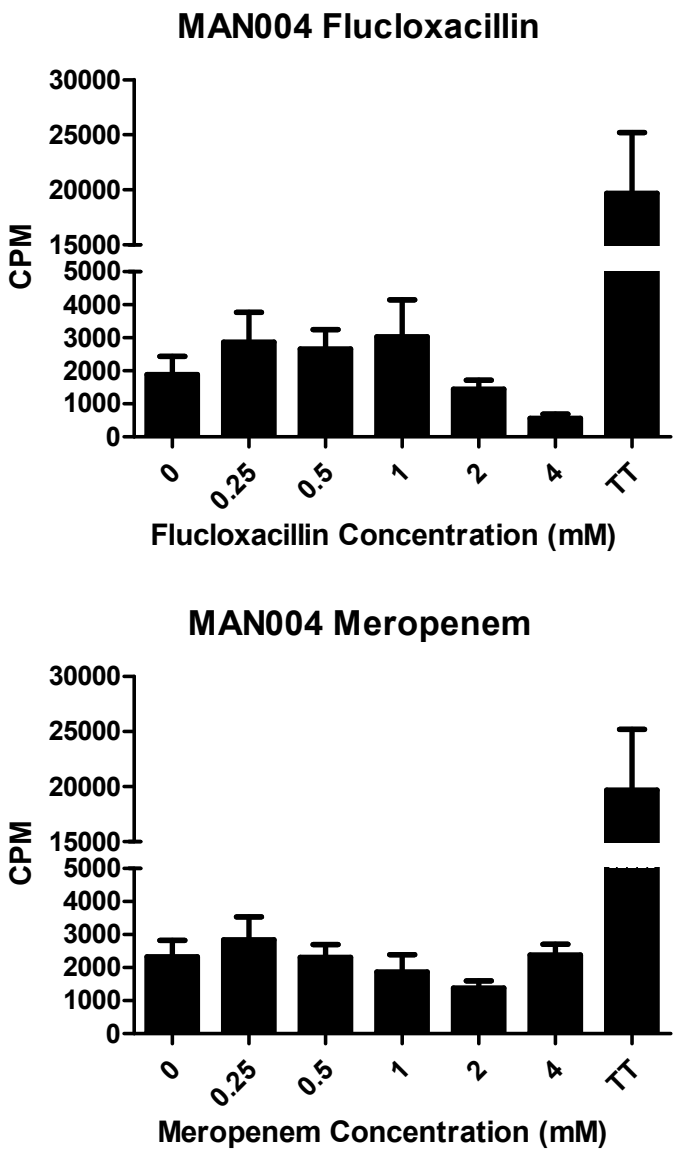


Figure 5.4 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5μCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

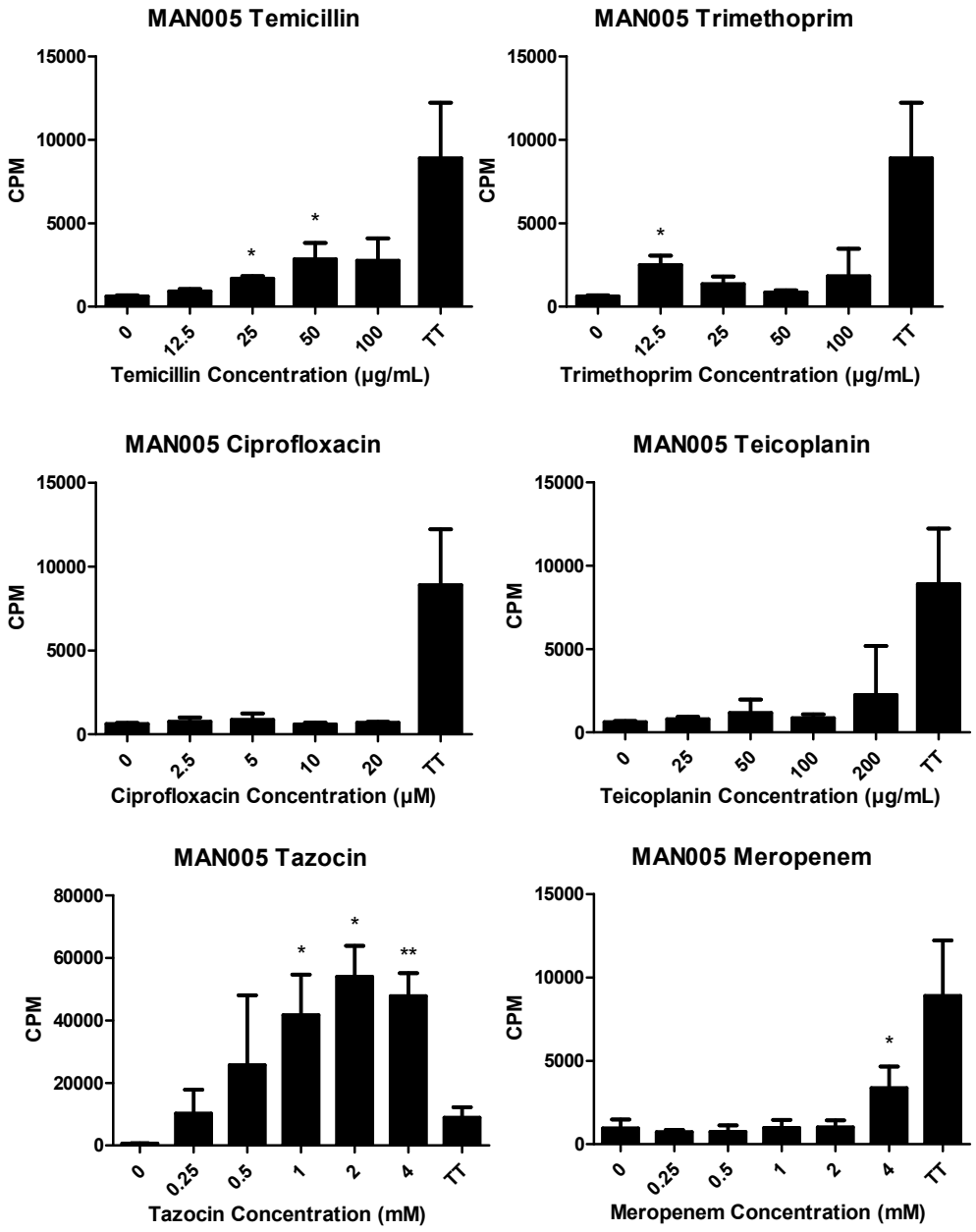


Figure 5.5 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5µCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

5.4.7 LTTs ON PAEDIATRIC PATIENTS PBMC TREATED WITH BOTH MEROPENEM AND TAZOCIN

Upon completion of the MAN cohort samples, we received CF paediatric blood samples from a new cohort RMCH. Both of the first two samples received from this new cohort (RMCH001 and RMCH002) were treated with both tazocin and meropenem. In each of these patient samples, a cellular proliferative response was confirmed by mitogen stimulation, but both tazocin and meropenem did not induce a significant immune response (measurable by LTT assay) at any concentration tested in either of the two patients. Tazocin did show a marginal increase in proliferation at 0.5, 1 and 2mM concentration in RMCH001, but replicates leading to large SD meant these were not deemed significant by statistical test (T test). However, stimulation index (SI) was calculated by: average of drug treated wells/average of negative control wells, showed a reasonably strong response (SI=3).

5.4.8 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH BOTH FLUCLOXACILLIN AND CIPROFLOXACIN

RMCH003 was another patient being treated with more than one antibiotic, this time flucloxacillin and ciprofloxacin were used. Mitogen stimulation again confirmed correct immune function of the cells, and 0.5mM flucloxacillin treatment led to the LTT detection of a significant increase in proliferation whilst ciprofloxacin treatment did not register and significant change.

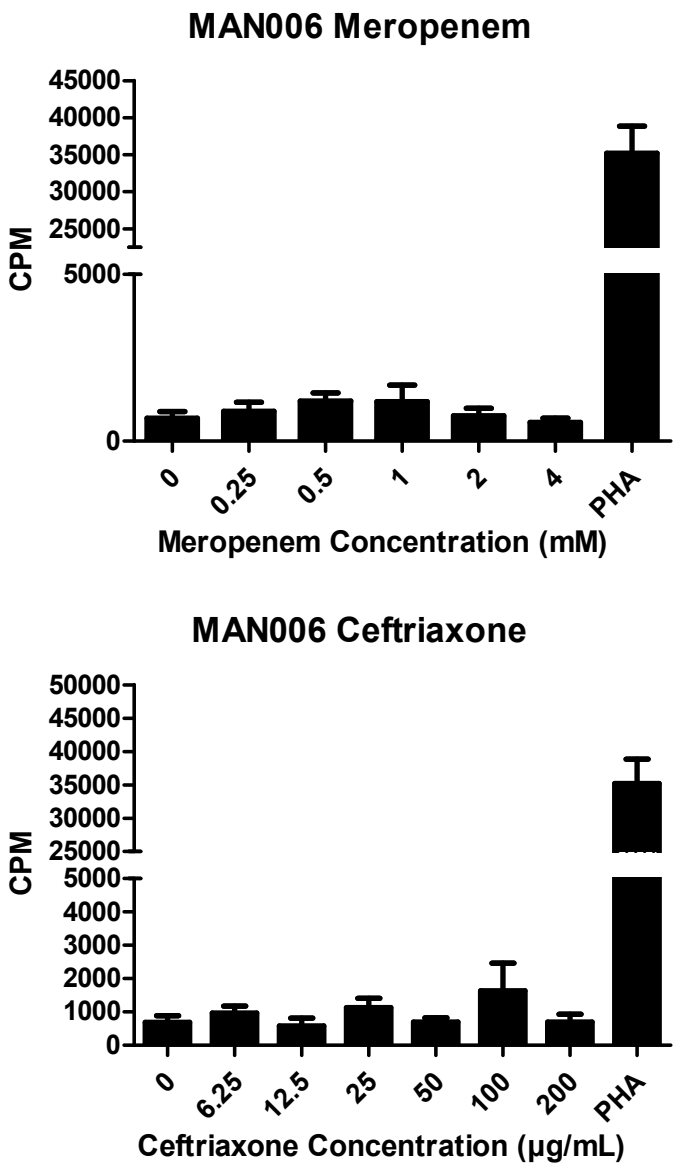


Figure 5.6 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5µCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

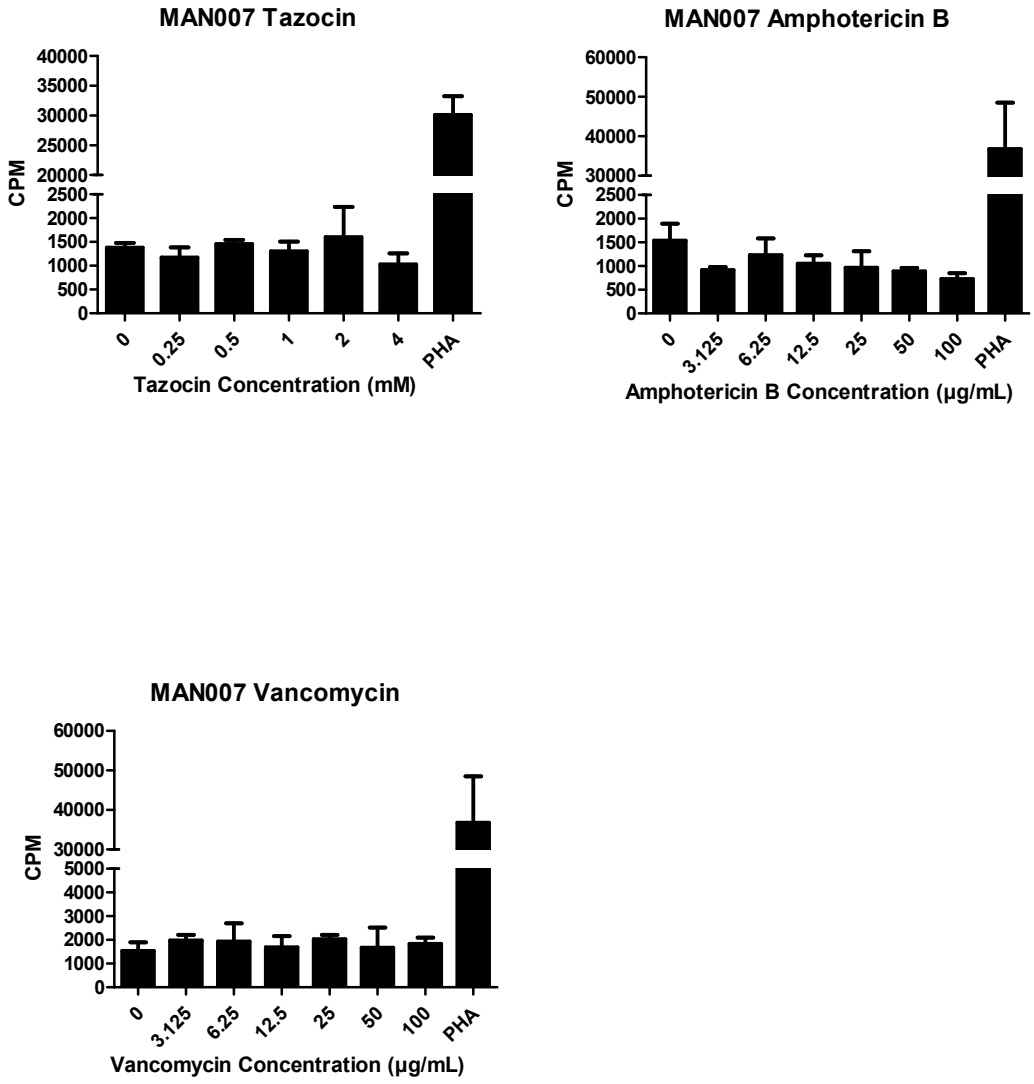


Figure 5.7 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5µCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

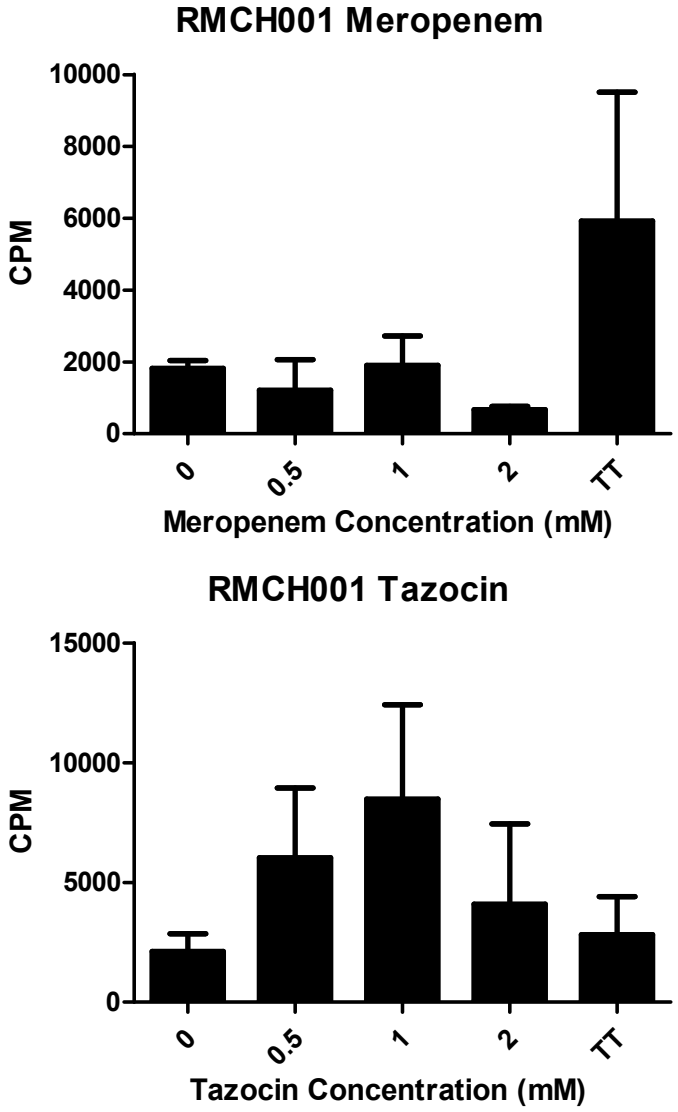


Figure 5.8 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5μCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

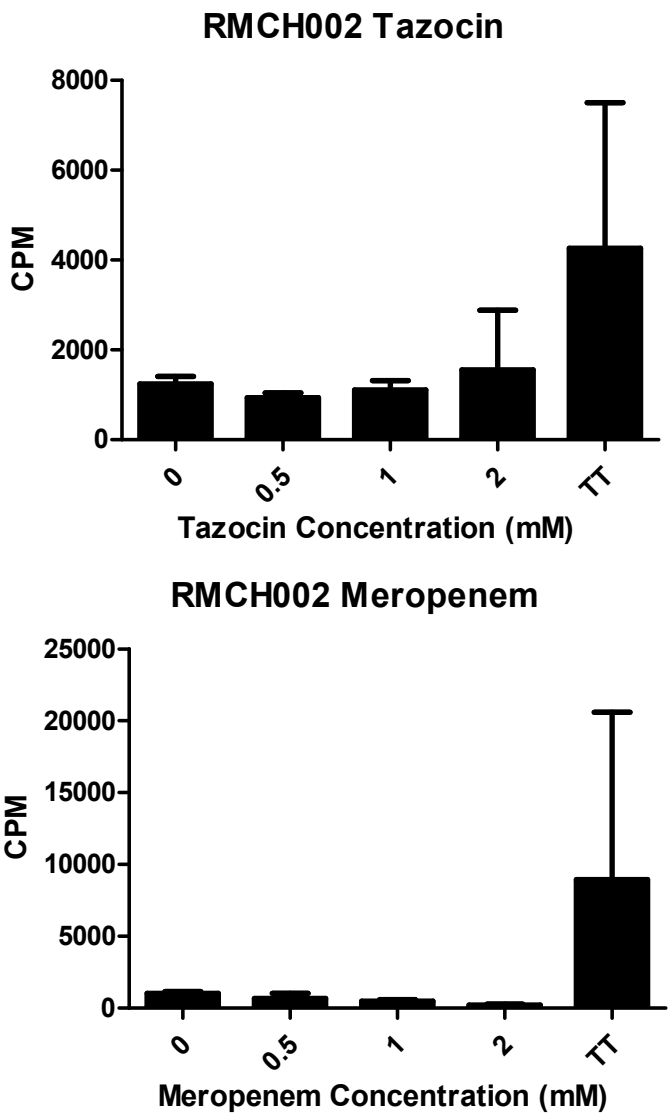


Figure 5.9 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5μCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

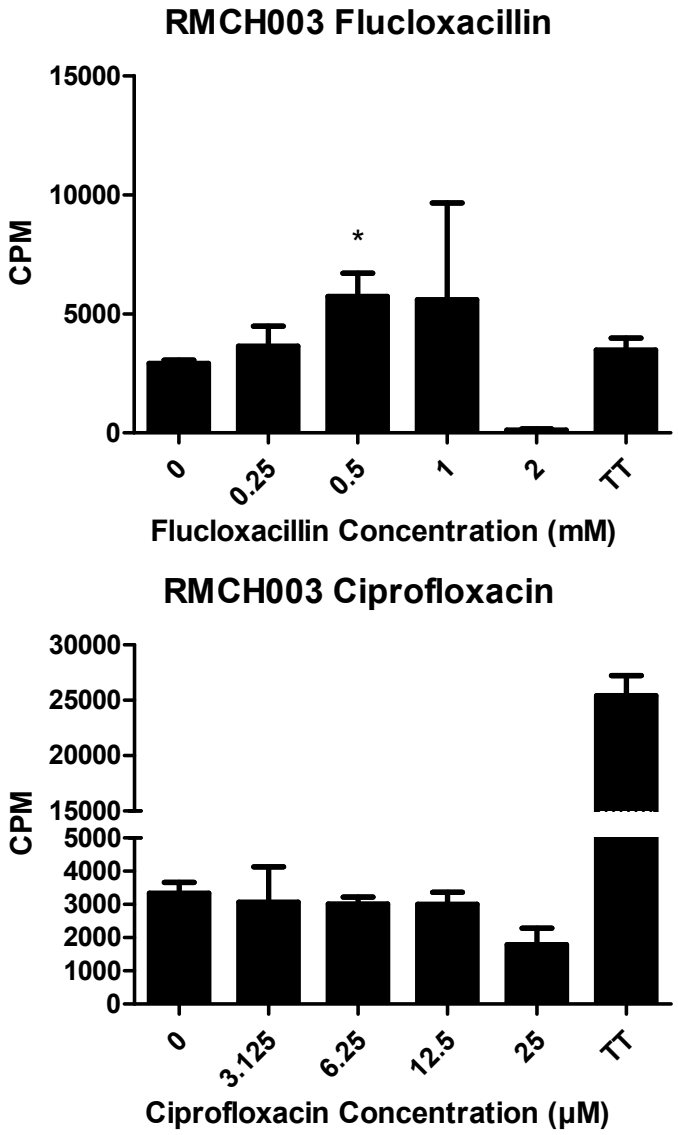


Figure 5.10 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5µCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

5.4.9 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH: AMOXICILLIN, CO-AMOXICILLIN, TEICOPLANIN AND TAZOCIN

Patient RMCH004 in *figure 5.11* was being treated with four different antibiotics: amoxicillin, co-amoxicillin, teicoplanin and tazocin. Despite attempts to outline the suspected culprit drug responsible for the purported adverse event, none of the drugs at any of the concentrations assayed resulted in an observed significant increase in proliferation. It must be noted that small (but not statistically significant) increases in CPM were observed at 1mM tazocin, and 50µg/mL teicoplanin treatments.

5.4.10 LTT ON PAEDIATRIC PATIENT PBMC TREATED WITH BOTH TAZOCIN AND AZTREONAM

The last patient sample received was RMCH005 (*figure 5.12).* In this final sample the patient was being treated concurrently with tazocin and aztreonam for CF induced lung and airway infection. Aztreonam did not invoke a significant immune response at any of the tested concentrations, whilst the LTT assay displayed a large significant increase in proliferation at both 1 and 2mM. Indeed, proliferative response to tazocin almost matched mitogen stimulation of the cells in this instance.

Overall, 13 different patient samples were obtained during the course of this study and were analysed by LTT in order to attempt to diagnose the culprit drug involved in suspected DHR. Of the 13 patients involved, only two of them (MAN001 and MAN003) were receiving single antibiotic treatment, whilst the others were receiving concurrent treatment of two or more antibiotics. MAN005, MAN007 and RMCH004 were treated with 6, 3 and 4 antibiotics respectively. Over half (8) of the 13 patient samples involved in the study did not lead to the detection of the culprit drug as they were negative for all drugs tested by LTT at all concentrations assayed. At least one potential culprit drug was outlined in the other five patient samples tested as a

significant increase in proliferation was observed after drug treatment when compared to the negative control (no drug). MAN005 was the only sample in which significant proliferation was detected in more than one drug tested as 4 out of 6 assayed drugs led to a significant increase in proliferation (temicillin, trimethoprim, tazocin and meropenem).

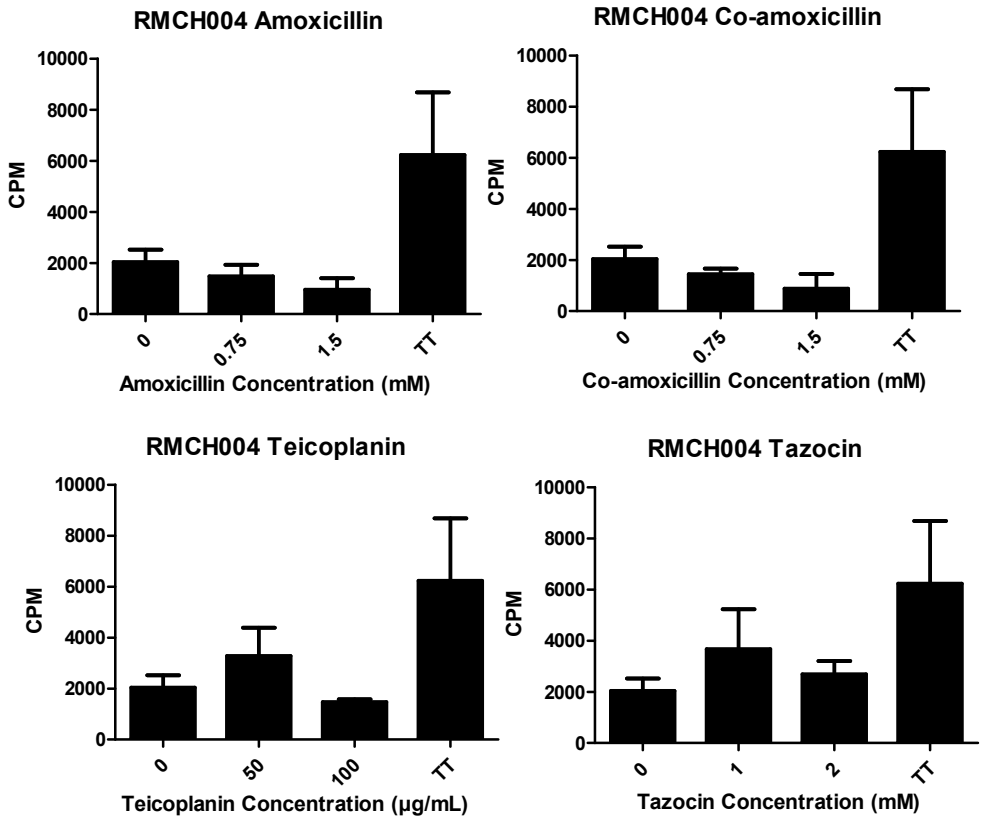


Figure 5.11 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5µCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

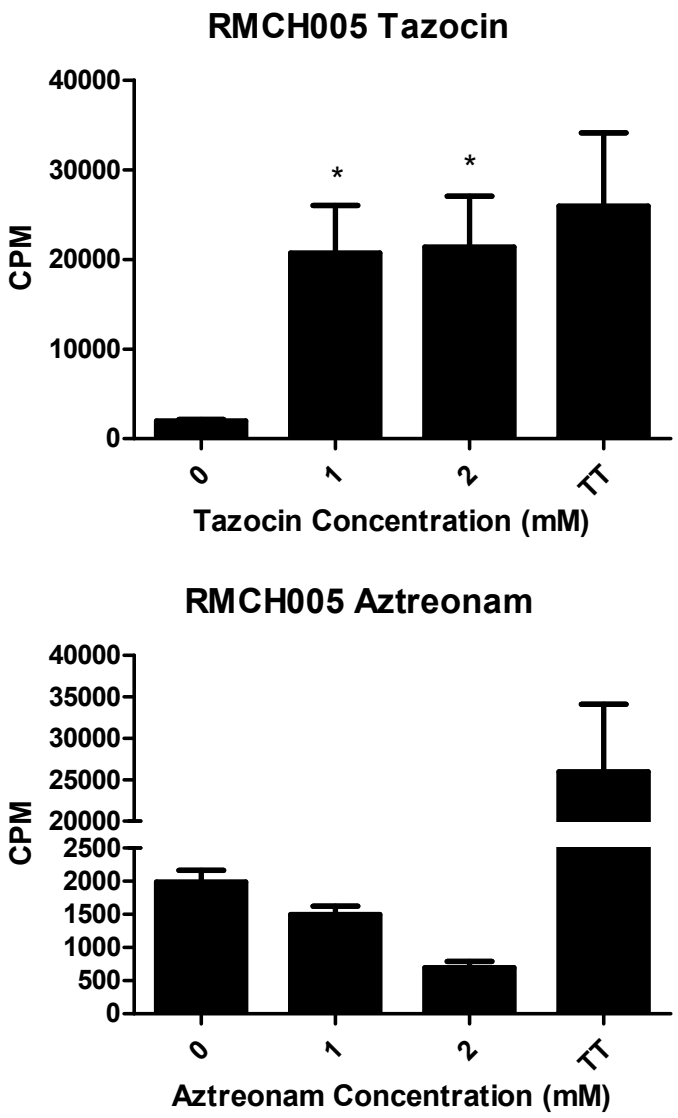


Figure 5.12 Diagnosis of culprit drug involved in DHR in patient LANC001 through use of LTT assay.

Paediatric patient blood sample was obtained to diagnose suspected DHR and detect culprit drug. Under sterile conditions blood was layered onto lymphoprep (1:1) and centrifuged, PBMC layer was removed through by pipetting before being washed and counted. Cells were then diluted in R9 media before being assayed in a 96-well plate at 150,000 cells per well in triplicate in 100uL volume R9. Patients' treatment drugs were then made up at a range of concentrations in R9 at a final volume of 100uL before being assayed in triplicate with the PBMC. Triplicates of no drug treatment (R9 only) or antigen and mitogen stimulation (TT or PHA) were also added. Plates were then incubated at 37˚c (0.5% CO_2) for five days. Sixteen hours before harvesting the plates 0.5μCi tritiated thymidine was added to all wells. Plates were then harvested and sealed by wax scintillation before CPM was calculated through counting of the plates on a beta-counter. Standard deviation was calculated and significant increase in proliferation was determined through paired T test when $p<0.05$.

5.5 DISCUSSION

Despite the prediction of DHR before occurrence being the ideal end goal through determination of the immunogenicity of a compound or drug in an individual, the problem of reliable diagnosis of DHR in patients who are already afflicted remains unsolved. *In vivo* testing can work effectively through skin patch and challenge testing to diagnose alleged DHR. Well trained clinicians can provide these tests in a semi-controlled environment to provide a more direct proof of the clinical relevance of the adverse manifestation of the treatment [304]. However, these *in vivo* tests can be both invasive, and risky depending on the severity of the DHR that individual suffers in response to drug challenge; this is exacerbated in children. Nevertheless, drug provocation testing has remained the gold standard even in paediatric testing [305], and for this reason, we believe that although adult work-ups of diagnostic testing can also be clinically relevant when dealing with a paediatric cohort of patients, this is not sufficiently reliable enough in adult testing, and even more so in children. A dependable *in vitro* test with both high sensitivity and specificity that works across a range of drugs (in this instance classes of antibiotics) should be the new gold standard, particularly when dealing with a group of patients that are so vulnerable to severe DHR due to long-term treatment regiments of multiple high dose antibiotics. The data obtained in this set of experiments will be used to assess the utility of the LTT both in the context of this set of patients, and in general use. It has already been made clear that there is an inherent need for an assay such as the LTT; it will be decided whether or not the current version of this assay is fit for purpose, before potential improvements to the assay will be suggested for future studies upon receipt of further patient samples.

As can be seen from the results section, over half of the patient samples analysed did not elicit a significant proliferative response to any of the drugs tested at any

concentration. In each of these instances different drugs were used from different classes of antibiotics. LANC001 was being treated for DHR in response to flucloxacillin and benzylpenicillin treatment, whereas MAN003 was treated solely with rifampicin. MAN004 was tested against flucloxacillin and meropenem like LANC001, and MAN006 was the first LTT negative patient not receiving flucloxacillin as they were being administered meropenem and ceftriaxone. MAN007 was actually negative to all three tested drugs (tazocin, amphotericin B and vancomycin). RMCH001 and 002 were negative in treatment to tazocin and meropenem whilst testing against amoxicillin, co-amoxicillin, teicoplanin and tazocin all yielded negative results in RMCH004. Previous studies have shown that whilst the sensitivity of the LTT is not usually high (up to 70%), the specificity of the assay is usually good (>80% up to 100% dependent on drug tested) [241,306]. It could be said that the negative results of the LTT indicates that the patients were not actually suffering DHR in response to any of the tested antibiotics used in the treatment of their disease. Indeed, misdiagnosis and self-diagnosis in response to an observable rash is still a major problem in the treatment of children. Many rashes seen in children that can be incorrectly diagnosed as DHR could actually be due to viral infections, which are common in children [293], or excipients [307]. Other infections and complications caused by the aetiology of CF such as lung or airway complications such as protein-energy malnutrition leading to dermatitis and other skin conditions [308,309]. It is likely that the LTT in this instance has correctly established that the skin reactions observed in the children are not DHR due to treatment with the tested antibiotics. However, it must be stated that this cannot be comprehensively proven by LTT alone, as there is still a chance that due to the specificity of the LTT [26,297], there is still a 1 in 5 chance that these data could be false negatives. With patient safety being of the upmost

importance, we could say that it is likely that these drugs tested by LTT are not causing DHR, but proceed with caution, as high morbidity manifestations of DHR can still occur if false negatives are present in the test.

Pleasingly, in this study the LTT did detect a statistically significant increase in proliferation to a single suspected culprit drug (out of two or more drugs tested) in four of the patients tested (LANC002, MAN001, RMCH003 and RMCH005). It is imperative that any *in vitro* test can elucidate the culprit drug in a multi-drug treatment strategy in order to know which drug must be removed from the treatment plan, whilst keeping the other drugs included. In the four patients listed above this was able to be achieved by the standard LTT assay used, and clinicians were informed which drug was the likely culprit in the suspected DHR observed.

For MAN005, four out of six drugs tested by the LTT resulted in a significant positive proliferative response. Multi-drug hypersensitivity (MDH) has been described in the literature; with some of the drugs featured in this experiment such as tazocin and trimethoprim being implicated regularly. It is stated that the effects of the second DHR can overlap with the remnants of the first drug stimulation [310]. Despite this, it is unlikely that all four out of these six drugs would raise a DHR in the patient if tested in confinement. This is an issue when the true culprit drugs for the DHR must be detected. MAN005 was being treated with drugs from the beta-lactam, penicillin, beta-lactamase resistant penicillin, carbapenem and semisynthetic glycopeptide classes of antibiotics. During multi-drug therapies such as this one, it can be difficult to single out or accurately detect one or possibly two drugs out of six due to the different immune mechanisms amongst different classes of antibiotics. Incidences of cross-reactivity between the different classes of antibiotics have been observed in DHR(up to 50% between penicillins and carbapenems for example), usually due to similarities

in side-chain determinants [311,312]. This may somewhat excuse the result of this LTT as potential cross-reactivity between these drugs has meant that more than one are responsible for the DHR, or that cross-reactivity has led to false positives in this particular LTT. The issue is that if the results of this LTT are false-positives, it is a dangerous precedent to set if four out of the six tested drugs are removed from the treatment plan for MAN005. It is clear that further understanding of drug reactions and cross-reactivity will both improve treatment plans for patients, lowering the risk of DHR, but also may help optimise the assay in order to circumvent the issue of false positives arising from antibiotic cross-reactivity [313].

Instances such as those above illustrate why although the LTT has its uses, and is the best *in vitro* test currently available for diagnosis, it is not truly fit for purpose and must be optimised further if we are to improve patient outcomes when dealing with groups of people at risk of severe DHR. This is a sentiment that has been echoed preciously [26]. Paediatric CF cohorts are heavily reliant on accurate diagnosis of DHR, as false positives leading to incorrect withdrawal of a drug can have dire consequences as treatment options become increasingly limited, resulting in the patient succumbing to lung and airway infections. It is for this reason that it is imperative that any assay in use can accurately pick out the culprit drug(s) from a multi-drug treatment – something that is not currently readily available. Similarly important is the ability to correctly diagnose DHR so that the use of problem drugs inducing DHR is not continued as severe DHR can occur in these patients leading to high morbidity and even mortality. Below are some suggested improvements to current *in vitro* strategies, as well as improvements to the LTT itself.

The ELISpot is an important *in vitro* tool to assess the cytokine secretion profile of immune cells following drug stimulation. It has been incorporated into diagnostic

testing as whilst LTT provides information on proliferation of the immune cell populations, ^{3}[H] thymidine incorporation does not show drug-specific reactive T cell proliferation explicitly, as proliferating bystander cells are also registered on the beta counter. The use of a negative control (no drug) aids in determining drug-specific proliferation; but the ELISpot can detect drug specific cytokine secretion in response to treatment and can also provide information on the nature of the immune response through knowledge of the predominantly secreted cytokine (TH1 – IL-2, IFN-γ / TH2 – IL-4, IL-13) [314,315]. The LTT generally has displayed higher specificity than the ELIspot, whereas the ELISpot can be more sensitive depending on the cytokine being detected; lower for IFN-γ, higher for IL-4 [300]. It would be useful to have both proliferation data, and antigen-specific cytokine secretion when determining a drugs immunogenicity and likely involvement in DHR. This is something than could be utilised in adult patient samples. For the diagnosis of DHR in children this is not currently viable due to the small volume of blood able to be obtained from children and the low PBMC numbers isolated subsequently, coupled with the high numbers of cells needed for an ELISpot (minimum 300,000 per well). We attempted to overcome this issue in the lab so that we could perform LTT and ELISpot concurrently by titrating the number of PBMC used per well. When attempting to use fewer than 150,000 cells per well in the LTT, significant increases in proliferation disappeared, meaning it was not possible to lower cell numbers used in the assays. Instead of lowering cell numbers needed for the LTT and ELISpot, supernatant could be taken from the wells of the LTT and cytokine secretion detected by ELISA. The main issue with this method being that this only shows overall cytokine secretion on a well-by-well basis, whereas the ELISpot can determine antigen-specific cytokine secretion on a cell-by-cell basis, determining the approximate number of cells secreting cytokines.

The improvement of the LTT lies in the question: 'are we replicating the current immune micro-environment in the wells of the LTT'. Can we learn anything from the mechanistic evaluation of naïve T cell priming from the previous experimental chapters, including immune regulation (signal 1: signal 2 axis) in order to more accurately replicate the correct immune micro-environment in the LTT. And by doing so, could we potentially improve the sensitivity and specificity of the assay for both adult patient diagnosis, but more importantly paediatric patients where problems surrounding low cell numbers from blood donations are apparent.

T regulatory cells (Tregs) can be involved in both pro- and anti-inflammatory responses to antigens [316], but are more frequently associated with immune suppression to maintain self-tolerance [317,318]. If regulatory T cells could be safely removed from PBMC population before and during drug incubation time (as we know from chapter 4 that numbers of Tregs increase during T cell priming) to discover immune responses that were previously suppressed by Tregs. However, preliminary experiments in chapter 4 showed that removing Tregs during drug treatment of immune cells in assay (by MACS separation) destroyed any proliferative response observed and the end point of the experiment; needs optimising further. In a similar way to the removal of T cells, the modulation of the co-signalling factors PD-1/PD-L1 and CTLA-4 can be used in order to more accurately replicate the correct immune microenvironment. Previously published studies from within the group [137,182] as well as data from chapter 4 of this thesis have shown that the co-signalling pathway plays an important role in both naïve and memory responses to drug challenge, and have already developed an easy method to modulate these pathways through blocking antibodies. The utility of PD-1/PD-L1 and CTLA-4 blocks should be explored in the LTT to determine if the

modulation of signal 2 in this way could (similar to Treg removal) uncover immune responses suppressed by negative regulation of signal 2.

The involvement of dendritic cells (DCs) in the immune response (both naïve and memory) is well-established. DC from hypersensitive patients can be driven by antibiotics such as amoxicillin to shift toward a semimature phenotype [319]. This information could be useful as we could isolate DCs from patients with suspected hypersensitivity and culture them with the different suspected drugs and then analyse the DCs for shift towards maturity (upregulation of MHCII) in order to determine which is the likely culprit drug prior to addition to the LTT as a source of antigen.

Implementing the above ideas into the LTT may improve the proliferative readout of the diagnostic assay, but another approach would be to use a different readout altogether. Upregulation of miR in response to adverse events such as liver injury has become an important research area for biomarker discovery [320]. Upregulation of miR and their role as regulators in immune response have also been observed [321,322]. Using this knowledge, the final experimental chapter of this thesis (chapter 6) will explore the relevance of this information through the alteration of an LTT-like diagnostic assay where the readout is miR expression. The modified assay will calculate upregulation of certain immunologically relevant miRs and its use as a potential novel diagnostic platform to improve on the LTT will be evaluated.

The main limitations of this study were the fact that the samples were anonymised so that we did not have any information on the genotype of the patients (who may express risk alleles for DHR to some of the drugs tested). It also meant that there were no *in vivo* confirmatory tests to compare the LTT results with, and we did not know the outcome of the patient treatment (whether drugs were removed from treatment and if this prevented further DHR. Despite this, the main aims of the chapter were achieved

in that we were successfully able to use these important paediatric samples in order to assess the utility of the LTT in instances such as this one. Whilst there were some positives, the data in this chapter suggests that the LTT test alone is not sufficient for use as a gold standard assay. The data in this chapter would have also been aided by some clinical data and observations. However, due to the nature of paediatric samples, we were not able to obtain this information. The data then gave rise to ideas on how we can improve the LTT (or develop new techniques) to develop desperately needed reliable *in vitro* diagnostic assays for DHR in both adults; and especially, children.

6 ASSESSMENT OF UP-REGULATION OF DIFFERENT T CELL ACTIVATION ASSOCIATED MIRNAS IN CD4+ T CELLS TO EVALUATE POTENTIAL AND SUSPECTED HYPERSENSITIVITY IN PATIENT SAMPLES.

6.1 INTRODUCTION

Adverse drug reactions (ADR) account for almost 6% of all hospital admissions causing approximatively 42,000 deaths every year in the European Union with an estimated economic loss of around 79 billion euros [323,324]. Drug hypersensitivity reactions, although rare, are one of the most serious forms of ADRs. Recent analysis of computational integrative data has estimated that a single drug could directly modify 25 transition immune states. We hypothesise that some of these changes may lead to human iatrogenic diseases that cannot currently be predicted during drug development [325].

The presence of drug-specific T cells in the peripheral blood and inflamed tissue of hypersensitive patients has been widely documented [111,326,327]. Several factors including genetic background, co-morbidities at the time of the reaction, metabolism and reactivity of the drug, individually or together, contribute to the generation of the drug-specific T cell response. Despite the complexity of the major histocompatibility complex (MHC), drug−T cell receptor (TCR) interaction, it has been possible to elucidate three basic mechanisms of drug-specific T cell activation through *in vitro* experiments using PBMCs from hypersensitive patients: (1) the hapten/pro-hapten pathway; (2) the pharmacological interaction or P−I theory; and (3) the altered self-repertoire pathway (as described in chapter 1) [274,328]. Furthermore, the ability of drug specific T cells to damage target tissue such as liver and skin has been described [149,155]. In the previous chapter, we showed that current *in* vitro diagnostic tests such as the LTT, whilst being the best available choice for diagnosis of DHR patients exposed to multiple possible culprit drugs, are not truly fit for purpose amongst all patient groups. The data acquired from the analysis of paediatric CF patient samples did not consistently enable us to diagnose DHR or pick out a single drug from multiple drug

treatments. This is problematic as incorrect drug withdrawal is potentially as big an issue as continued use of a drug causing an adverse event, especially for paediatric cystic fibrosis patients. That said, the LTT is the most consistent assay for the diagnosis of DHR across a range of drugs from different classes, especially when compared to alternatives such as the ELISpot and CD69 up-regulation assays. Assays such as the LTT and ELISpot are adequate assessors of immunological responses by through measurement of proliferation of cells and cytokine secretion respectively. As suggested at the conclusion of the previous chapter, whilst these current readouts for assessing immune response to drug challenge have their uses, it is important to understand the what is happening at the transcription and post-transcription level, as immune regulation occurs at this stage, leading to suppression and activation of immune responses. Another aspect of the LTT that can be improved upon is the length of time taken to perform the assay (6 days) – in such a time sensitive situation where the DHR must be rapidly diagnosed in order to prevent potential death of the patient. In this chapter, one of the initial aims was to investigate the role of transcription and post-transcriptional factors in the development of DHR, as well as improve the time taken to perform any novel *in vitro* diagnostic test, developed through the knowledge gained in this set of experiments.

Little is known about the role of post-transcriptional regulators, particularly miRNAs, in the pathogenesis of drug hypersensitivity. MiRNAs are single stranded 22 nucleotides RNAs that regulate gene expression by binding to the 3′ untranslated region (UTR) of their target promoting translational repression or the direct degradation of the mRNA. Up-regulation of a specific miRNA promotes higher inhibition of the target gene which manifest in phenotypic and physiological changes in the cell [208]. MiRNAs are involved in regulating the expression of 30-50% of genes;

and so are involved in many key physiological and fundamental cellular processes [209]. They can elicit regulation that alters the concentration of key protein components of cascading signalling pathways of immune activation at the post-transcriptional level and importantly, studies have shown that genetic ablation of miRNA machinery has led to auto-immunity [210]. MiRNAs are becoming increasingly notorious as key regulators of key biological processes; proliferation, differentiation and development, as well as crucial regulators of both innate and adaptive immune responses [211] through a purported role in the induction, maintenance and function of Tregs, and regulating the differentiation of APCs dendritic cells and macrophages through TLRs [212].

Over a hundred miRNAs have been shown to be expressed by immune cells and have an impact in basic immunological process. Despite the wide variety of miRNAs showing an altered expression, only a few have been studied in detail. These include miR-9, miR-17 ≈ 19 cluster, miR-21, miR-146a, miR-155, miR-181a, and miR-214. For most of these, their target genes have been validated and their function has been evaluated during T cell activation by functional assays [214–220].

Interestingly, the role of miRNA in immune function regarding the development of DHR can also tie in well to the data obtained in chapter 4 – as miRNA can act as post-transcriptional regulators of other immune regulatory pathways that are discussed through the course of this thesis. This includes the aforementioned regulation maintenance and function of Tregs, in addition to involvement in the co-signalling pathway; PD-L1 specifically. miRNA binding to PD-L1 mRNA on APC can lead to degradation and ultimately suppression of the ligand and therefore overall suppression of the PD-1/PD-L1 axis leading to increased immune activation; with miR-513, miR-570, miR-34a, and miR-200 reportedly displaying inverse correlation to PD-L1

expression [221]. MiRNAs have also been directly implicated in DHR through targeting and post-transcriptional regulation of the granulysin gene [222].

Regarding drug hypersensitivity reactions, microarray analysis has been made using biopsies of patients with mild and severe cutaneous reactions, and some of the miRNAs, selected for this study showed an altered expression. In particular, a direct correlation between miR-18a expression and the severity of the reaction was observed. Moreover, bioinformatics and functional analysis demonstrated that miR-18a targets the BCL2L10 gene, a negative regulator of apoptosis. Interestingly, the same miRNA is up-regulated during CD4+ T cell activation and has a similar effect on CD4+ T cells, but only when it is overexpressed alone [217,329].

The main challenge associated with this chapter was developing an assay that would also overcome the main pitfalls associated with current *in vitro* assays such as the LTT and ELIspot. Whilst we were not able to obtain further paediatric samples in order to carry out these studies, it was apparent throughout the project that any assay developed during the course of these set of experiments must improve upon the aforementioned LTT, not only for adult patient populations, but for vulnerable paediatric patients as well. The assay utilised in this chapter improved upon the time taken to perform analysis from six days to three; speeding up time taken for diagnosis. We were also able to ensure that the miRNA assay did not use more cells than the LTT, and used fewer cells than needed for the ELISpot.

The aim of this study was to determine the changes in the expression of miRNAs previously shown to be involved in CD4+ T cell activation (miR-9, miR-18a, miR-19b, miR-21, miR-155 and miR-214) using drug-specific CD4 + T cell clones and PBMCs obtained from drug hypersensitive patients. Should we discover up-regulation of immunologically relevant miRNA listed above in response to specific drug

challenge, the most important question to be answered if we wish to further develop this knowledge into a viable *in vitro* DHR diagnostic assay is: are we correlating drug specifically induced, increased expression of targeted immunologically relevant miRNA for DHR with proliferative responses observed in functional T cell assays?

6.2 CHAPTER AIMS

1. Assess the drug-specific up-regulation of DHR immunologically relevant miRNA in hypersensitive patients in response to drug challenge.
2. Determine if up-regulation of miRNA observed correlates to a proliferative T cell response.
3. Assess whether or not miRNA upregulation assays used in this chapter are a feasible improvement to the classical *in vitro* diagnostic assays such as the LTT and ELISpot.

6.3 METHODS

All methods for techniques seen in this chapter can be found in detail in Chapter 2: Methods and Materials.

6.4 RESULTS

6.4.1 ACTIVATION OF CD4+ T CELLS WITH PIPERACILLIN AND CARBAMAZEPINE

A total of 11 CD4+ T cell clones from carbamazepine and piperacillin patients were used. IFN-γ/IL-22 and IFN-γ/granzyme-B were selected as relevant ELISpot readouts for piperacillin and carbamazepine respectively, based on recent investigations [50]. All clones were stimulated to proliferate and secrete cytokines/cytolytic molecules after drug-treatment (Table 6.1 and *Figure 6.1*). All TCC secreted IFN-γ following drug treatment. Four out of five piperacillin-specific TCC secreted IL-22, while all of the carbamazepine-specific TCC secreted granzyme-B.

6.4.2 DRUG-SPECIFIC CD4+ T CELL CLONES UP-REGULATE PARTICULAR SETS OF MIRNAS

Expression of miRNAs in activated TCC was measured by qRT-PCR at 24h when incubated with antigen presenting cells (APC) in the presence or absence of the relevant drugs. A drug-specific increase in miR-155 expression was observed with all clones. MiR-18, miR-21, and miR-9 showed up-regulation in 10, 7, and 6 of the piperacillin and carbamazepine clones (*Figure 6.2*. A, B). Only two carbamazepine TCC showed up-regulation of miR-19b and miR-146, whereas piperacillin TCC did not up-regulate these miRNA. Increased expression of miR-214 was not detected. A strong positive correlation was observed between the extent of proliferation and miR-155 (R2 = 0.85) and miR-18a (R2 = 0.74) expression in carbamazepine clones (Table 6.1).

Table 6.1. Proliferation, IFN-γ secretion, and miRNAs expression by drug-specific CD4+ T cell clones obtained from piperacillin and carbamazepine hypersensitive patients

			MiR-9		MiR-18		MiR-21		MiR-155	
TCC	SI	IFN-γ	no drug	drug	no drug	drug	no drug	drug	no drug	drug
PIP #1	10.83[a]	+++[b]	0.37	6.17 ± 1.68[c]	0.02	84.03 ± 12.7	0.39	8.29 ± 1.43	0.73	871.6 ± 47.08
PIP #2	16.93	+++	0.6	3.86 ± 0.89	0.57	5.87 ± 2.87	0.69	7.79 ± 2.64	0.01	2.68 ± 1.00
PIP #3	8.77	+++	1.23	8.02 ± 2.72	1.56	1.04 ± 0.01	3.64	32.16 ± 4.05	1.01	47.77 ± 13.49
PIP #4	3.54	+++	0.38	1.29 ± 0.08	2.03	114.5 ± 0.44	1.6	1.48 ± 0.82	1.29	3.5 ± 0.95
PIP #5	1.60	++	1.36	0.77 ± 0.01	0.67	1.61 ± 0.35	1.47	0.66 ± 0.02	1.59	9.18 ± 1.04
CBZ #7	18.00	+++	0.57	2.58 ± 0.35	0.54	438.8 ± 46.3	0.77	4.66 ± 0.46	1.25	105.9 ± 7.4
CBZ #8	1.74	+	0.22	3.47 ± 0.91	0.19	2.8 ± 0.62	1.24	14.14 ± 0.00	0.21	2.16 ± 0.56
CBZ #9	4.17	+++	1.09	0.28 ± 0.02	0.38	1.48 ± 0.33	0.24	0.51 ± 0.43	0.90	12.14 ± 3.38
CBZ #10	8.93	++	1.39	1.4 ± 0.31	2.46	14.91 ± 3.33	0.67	2.89 ± 0.46	1.88	24.65 ± 6.33
CBZ #11	13.89	+++	0.67	0.36 ± 0.14	0.41	6.53 ± 0.91	0.92	20.55 ± 4.66	1.19	21.83 ± 1.37
CBZ #12	1.70	+++	0.2	0.01 ± 0.00	0.86	1.49 ± 0.08	0.65	0.00 ± 0.00	1.59	2.61 ± 0.12

[a]Data presented as stimulation index (SI) calculated as cpm in drug incubation/cpm in control incubations. [b]Spot forming units (SFU) after piperacillin (PIP; 2 mmol/L) and carbamazepine (CBZ; 50 μg/mL) treatment: + , 10–30; ++, 30–100; +++, >100. [c]Relative expression (RE). All values are the mean of a triplicate ± standard deviation.

A similar relationship was not observed with the piperacillin clones with the same miRNAs; however, a moderate but positive correlation between the extent of piperacillin-specific proliferation and miR-9 expression (R2 = 0.51) was determined. To confirm that miRNAs were expressed by CD4+ T cells during drug challenge, we incubated APC and TCC in the absence of drug, and the expression of the miRNAs was measured a different time points. As expected, the RE values were below one in

most cases, though some TCC showed a slightly higher value, but when the same TCC was incubated in the presence of the drug, a positive 3 to 10-fold change was seen (Table 6.1, *Figure 6.2* A, B). Moreover, to rule out any change in miRNA expression because of a non-specific interaction of the drugs and other immune cell receptors, TCC and APC were incubated alone in the presence of the drug, and no change of miRNAs expression was observed (*Figure 6.2* D). Finally, to demonstrate that miRNAs up-regulation was the result of the initial signals provided by the drug to the TCR, we incubated the best drug responding TCC (clones that expressed all miRNAs with drug-treatment) with a non-toxic concentration of cyclosporine-A. A significant decrease in expression of all the miRNAs, below the reference value, was observed with cyclosporine-A. *Figure 6.2* C compares the response of clones to drug in the presence and absence of cyclosporine-A. Thus, our results demonstrate that miRNA up-regulation is the result of a drug-specific CD4+ T cell activation.

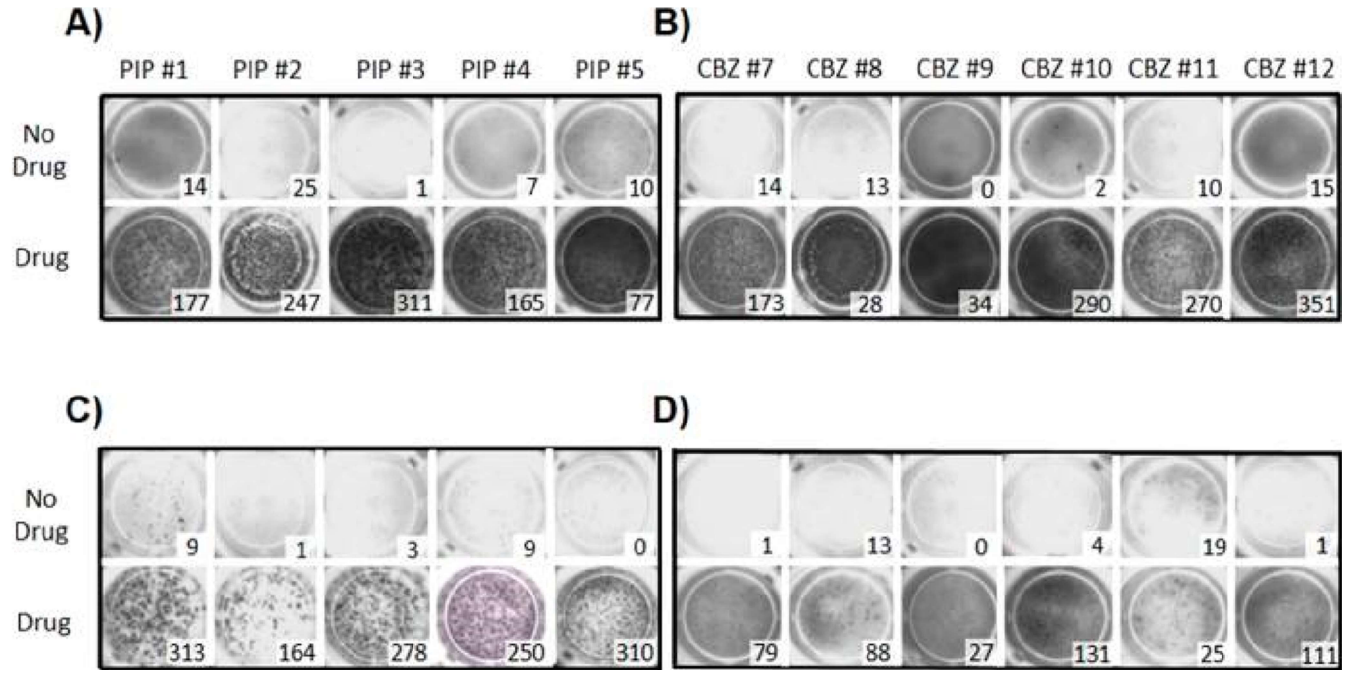


Figure 6.1 Cytokine secretion from activated drug-specific CD4+ T cells. (A) IFN-γ secretion from piperacillin-specific CD4+ T cell clones and (B) carbamazepine-specific CD4+ T cell clones incubated for 48 h with 2 mmol/L and 50 μg/mL of piperacillin and carbamazepine, respectively. (C) IL-22 secretion from piperacillin-specific CD4+ T cell clones incubated for 48 h with 2 mmol/L piperacillin. (D) Granzyme B secretion from carbamazepine-specific CD4+ T cell clones incubated 48 h with 50 μg/mL of carbamazepine. The number in the images correspond to the number of spot forming units (SFU) where a value above 30 SFU is considered positive.

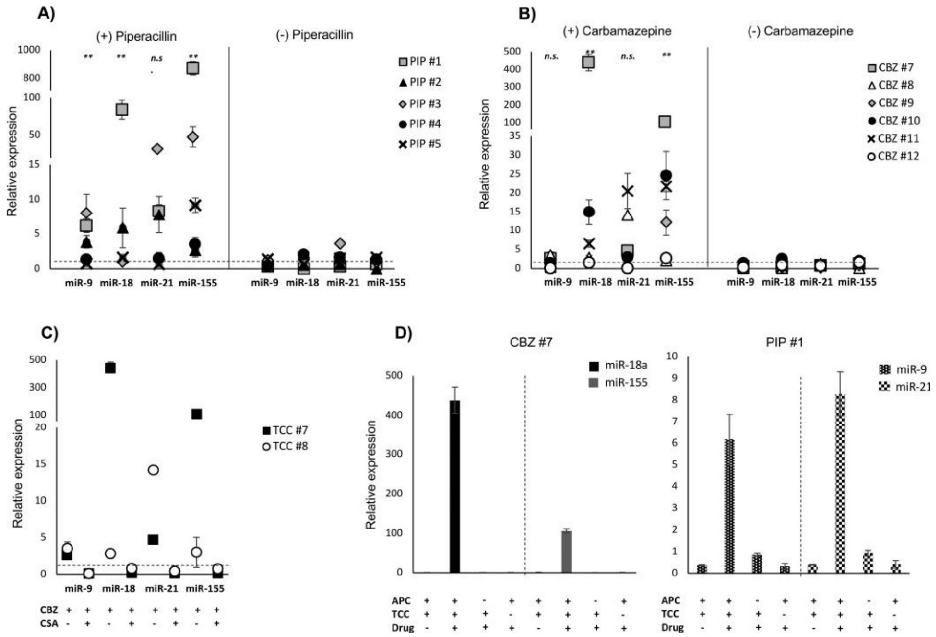


*Figure 6.2 Up-regulation of miR-9, miR-18a, miR-21, and miR-155 is the result of the interaction between APC, drugs, and CD4+ drug-specific T cells. (A) MiRNAs expression in piperacillin-specific TCC and (B) carbamazepine TCC in the presence or absent of the culprit drug. (C) Cyclosporine (1μg/mL)blocks the up-regulation of miR-9, miR-18a, miR-21, and miR-155 in two carbamazepine-specific TCC incubated with APC and the drug for 24h. (D) The presence of APCs, the drugs and TCCs are necessary for miRNAs up-regulation. Values are shown as relative expression and are the mean of a triplicate ± standard deviation. Statistical analysis was performed between relative expression of TCC cultured with medium alone and TCC cultured with drug. **P ≤ 0.05l n.s., Not significant.*

Table 6.2. Patient's clinical information and LTT results

patient	sex	age	drug	skin	time taking the drug (days)	co-morbilities	SI
SMX1	F	60	sulfamethoxazole/trimethoprim	MPE	10	RI	5.0
SMX2	M	35		MPE	9	RI	3.0
LMTG1	M	32	lamotrigine	TEN	7	MDD	2.0
LMTG2	M	40		SJS	12	MDD	2.4
CBZ1	F	47	carbamazepine	SJS	7	MDD	2.1
PIP1	F	33	piperacillin	MPE	7	CF	3.6
PIP2	F	29		MPE	8	CF	1.2
PIP3	M	28		MPE	11	CF	28.0

[a]Male (M) or female (F) hypersensitive patients to sulfamethoxazole (SMX 1-2), lamotrigine (LMTG 1-2), carbamazepine (CBZ 1), and piperacillin (PIP 1-3) with different skin clinical manifestations including maculopapular exanthema (MPE), toxic epidermal necrolysis (TEN), and Stevens–Johnson syndrome (SJS) were enrolled in the study. Patients were diagnosis with respiratory infection (RI), major depressive disorder (MDD), or cystic fibrosis (CF). Proliferation was measured as stimulation index (SI).

6.4.3 TIME-DEPENDENT UP-REGULATION MIRNA IN DRUG STIMULATED PBMC FROM HYPERSENSITIVE PATIENTS

To investigate if the miRNAs up-regulated in TCC are also up-regulated in PBMC of hypersensitive patients, we focused on miR-18a, miR-21, and miR-155. Sulfamethoxazole, lamotrigine, piperacillin, and carbamazepine hypersensitive patients presenting with a range of cutaneous manifestations, varying in severity from maculopapular exanthema to toxic epidermal necrolysis, were included in the study (Table 6.2). PBMC from all patients, but one, were stimulated to proliferate in the presence of the culprit drug (stimulation index of 2 or higher; Table 6.2). Because of the variety of skin manifestations, which might affect the number of blood circulating drug-specific T cells and their phenotype/function [330], miRNA expression was assessed at two time points, 24 and 48h. PBMCs from all patients, including the one with negative lymphocyte transformation test showed up-regulation of miRNAs after drug treatment (*Figure 6.3*).

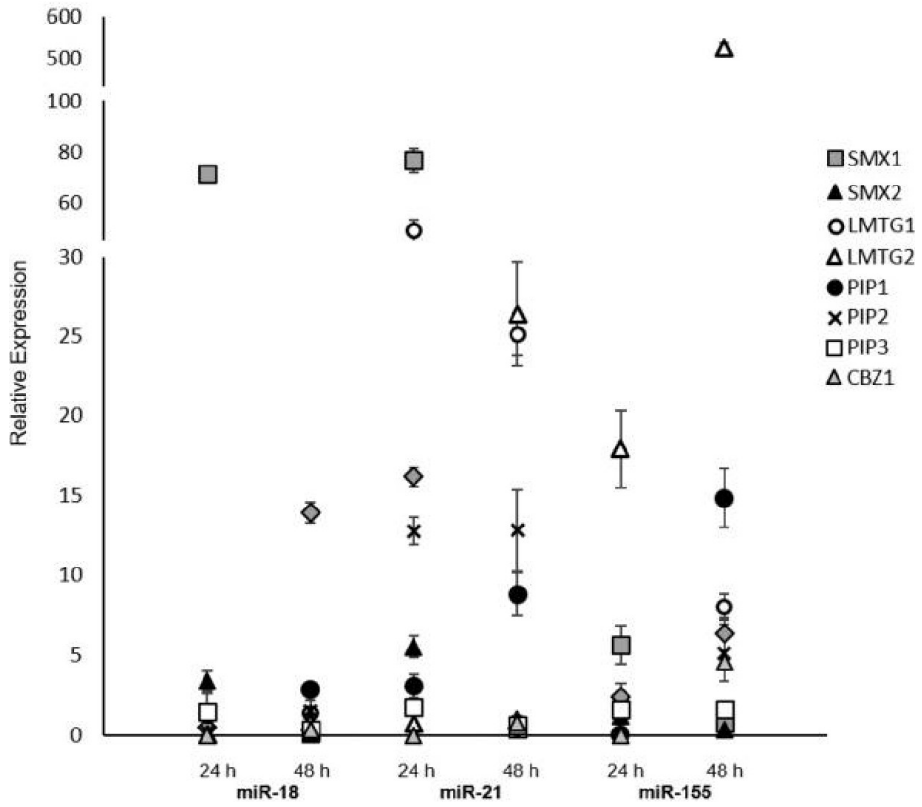


Figure 6.3 Time-dependent expression of miRNAs in PBMCs of drug hypersensitive patients. Relative expression of miR-18a, miR-21, and miR-155 from eight hypersensitive patients to sulfamethoxazole (SMX1-2), lamotrigine (LMTG1-2), piperacillin (PIP1-3) or carbamazepine (CBZ1) with different clinical manifestations. PBMCs were incubated in the presence of culprit drug for 24 and 48h prior to analysis. Values are shown as relative expression and are the mean of a triplicate ± standard deviation.

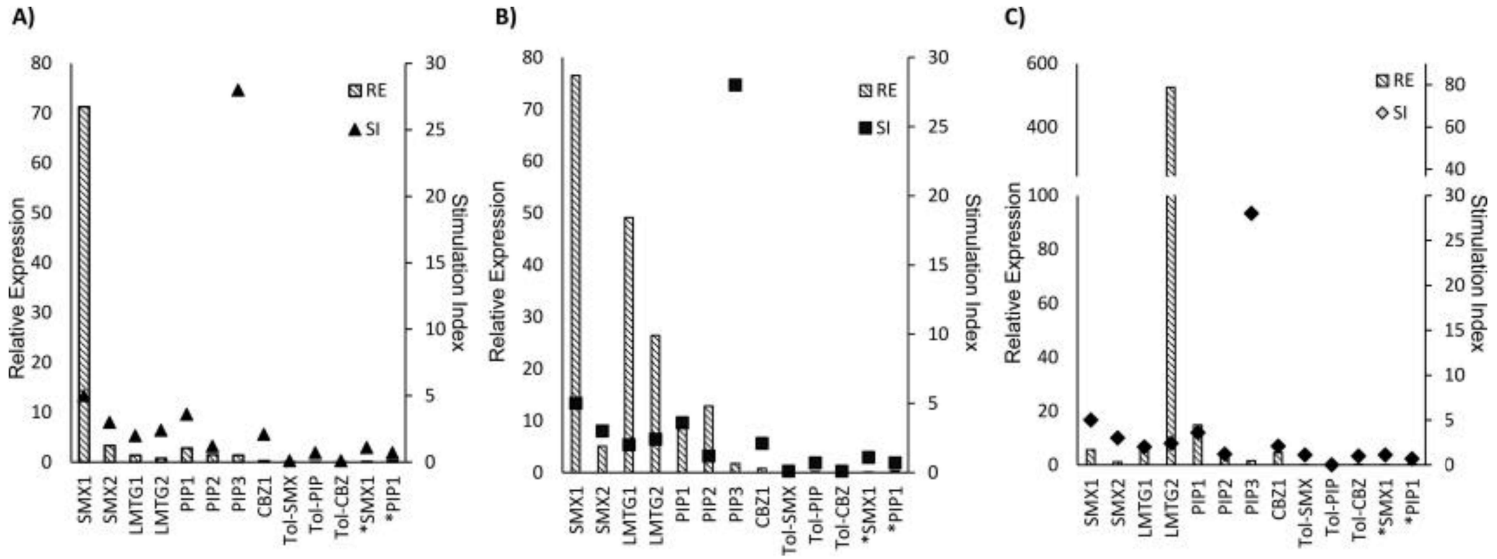


Figure 6.4 Maximal miRNAs up-regulation can be correlated with the degree of proliferation in the presence of the culprits' drugs. Relative expression of (A)miR-18a, (B)miR-21, and (C)miR-155, in dictated with the bars, in drug-treated PBMCs, shows that a positive expression of miRNAs correlates with a positive proliferation (SI), indicated with symbols, in hypersensitive patients to sulfamethoxazole (SMX1-2), lamotrigine (LMTG1-2), piperacillin (PIP1-3), or carbamazepine (CBZ1) with different clinical cutaneous manifestations. Importantly, drug treatment of PBMCs obtained from tolerant patients (Tol) or hypersensitivity patients in the absence of the drug (*) did not up-regulate miRNAs or stimulate proliferation.

As seen with the TCC, no increase in PBMCs miRNA was observed over the duration of the assay in the absence of the drug in the PBMCS (*Figure 6.4*). The times in which the different miRNAs were expressed with drug treatment differed. A higher level of miR-18a was detected in most patients after 48 h, while miR-21 was expressed earlier, at 24 h. In contrast, miR-155 was detected at a similar level at both time-points, irrespectively of the severity of the skin reaction. Drug-stimulated PBMCs from the SJS/TEN patients showed high levels of all the miRNAs analysed. *Figure 6.4* compares the lymphocyte transformation test results of individual hypersensitive and tolerant patients with the up-regulation of miR-18a, miR-21, and miR-155. All patients showed a positive expression of at least 1, in most cases 2, miRNAs. In some cases, drug-treated samples displayed 5−20 times higher miRNAs expression than basal levels. Up-regulation of miRNAs was seen with drug-treated PBMCs from piperacillin hypersensitive patients; however, a direct correlation between miRNAs upregulation and proliferation was not always observed. When the expression of miRNAs was assessed in piperacillin tolerant or other tolerant controls, the presence of the culprit drugs did not up-regulate the expression of any of the miRNAs tested (*Figure 6.4*).

6.5 DISCUSSION

Until recently, the main method of prediction, diagnosis, and analysis of DHR has been heavily reliant on functional T cell assays; with the end readout being either proliferation (LTT & T cell priming) or cytokine secretion (ELISpot/ELISA). It was decided that opposed to looking purely at proliferation or cytokine secretion, it would be important to have an understanding at what is happening at the transcription and post-transcription regulatory levels in the initiation of DHR. A great deal of attention has focused on the changes in gene expression, particularly miRNAs dysregulated during T cell activation, since they play an important role in the phenotype of the activated cells and neighbouring cells. T cell activation is paramount for the initiation of drug hypersensitivity reactions; however, until now, the regulation of miRNA expression following drug-specific T cell activation has not been addressed. Here, we have shown an up-regulation in the expression of several miRNAs, previously studied during T cell activation, in drug-specific CD4+ T cell clones and PBMCs from patients with different forms of cutaneous hypersensitivity.

Two sets of TCC were analysed to explore the different patterns of miRNAs expressed during drug-specific CD4+ T cell activation. Piperacillin-specific and carbamazepine-specific TCC were used because, at least *in vitro*, the drugs activate T cell receptors via hapten and PI pathways, respectively. With carbamazepine, the kinetics of T cell activation are too rapid for antigen processing to be a prerequisite [158,159]. Furthermore, inhibition of antigen processing does not alter the strength of the carbamazepine specific T cell response. After drug-specific CD4 + T cell activation, most of the selected miRNAs were up-regulated, and in certain circumstances their expression directly correlated with the strength of the drug-specific CD4+ T cell proliferative response. Thus, by directly comparing miRNA up-regulation and CD4+ T cell

proliferation, it was possible to establish the polarized pattern of miRNAs. Strongly proliferating carbamazepine-specific TCC showed the highest levels of miR-18a and miR-155 up-regulation, while miR-9 up-regulation was seen with the TCC proliferating strongly to piperacillin. MiR-19b and miR-146 up-regulation was not consistently expressed, only two of the CBZ clones showed positive values, whereas miR-214 was not up-regulated with any of the TCC. It is important to emphasize that although this pattern of miRNAs expression seems to be characteristic for the CD4+ T cells, further investigation is needed to explore the miRNAs pattern in drug-specific CD8+ T cell clones and in clones activated by other drugs.

Previous microarray studies have found a significant change in the expression of several miRNAs in biopsies and plasma of patients with SJS/TEN, where miR-18a, a member of the miR-17≈ 92 cluster family, showed the highest up-regulation [329]. Mechanistic studies using a miR-18a mimic transfected into keratinocytes revealed that the miRNA was involved in the induction of apoptosis *in vitro*. Interestingly, the same effect was seen in CD4+ T cells when the miRNA was artificially and individually transduced during CD4+ T cell activation. Therefore, it is possible that the apoptotic effect seen in drug activated CD4+ T cells is not evident because of the expression of other members of the cluster or the expression of miR-9, miR-21, and miR-155, which collectively promote proliferation. Therefore, if the effects of miR-18a are not seen directly in the drug activated CD4+ T cells, it is possible that the miRNA could target a different cell type. Nonetheless, our results showed that under certain circumstances drug-specific activation might contribute to the overexpression of miR-18a.

MiRNA communication between different cell types has been demonstrated in cutaneous contact hypersensitivity reactions where exosomes containing miR-150

travel in plasma delivering a suppressive effect on the inflammatory response [331]. Thus, it is necessary to determine the expression levels of miR-18a in micro vesicles secreted during drug challenge of different T cells repertoires, at different time points. Furthermore, it will be important to evaluate their effect on keratinocytes. Interestingly, carbamazepine CD4+ T cells showed the highest expression of miR-18a, and carbamazepine is associated with a high incidence of SJS/TEN [134]. Thus, it is possible that the activation of TCC with carbamazepine is related to the overexpression of miR-18a and together with other factors, such as genetic background, is a predisposing element for the development of severe bullous reactions.

MiR-155 was also highly expressed in TCCs, and besides its effect on proliferation, it plays an important role in the inflammatory response. Functional studies using miR-155−/− mice show a tendency to generate lower numbers of Th1 and Th17 cells during autoimmune diseases, and *in vitro* studies with CD4+ T cells from miR-155−/− mice secreted lower levels of IFN-γ when compared with the same cells from the WT animals [332]. Thus, we compared the selective expression of miR-155 with the secretion of IFN-γ after drug treatment. The level of expression with carbamazepine-specific TCC correlated with the level of IFN-γ secretion, although not in a linear manner, in four of the six clones tested, but this pattern was only seen in two of the five piperacillin TCC.

IL-2 is necessary for TCC survival, and its presence does not affect the expression of miR-21 and miR-155. In contrast, it might affect the expression of miR-9 which targets genes related to IL-2 expression [218]. Thus, to avoid any interference of IL-2 in our studies, the TCC were deprived of the growth factor 48h before stimulating the clones with either piperacillin or carbamazepine. To demonstrate that miRNA up-regulation

was the result of drug-specific CD4+ T cell activation and downstream signalling, an extra experiment was conducted where cyclosporine-A was added to the assay. The drugs did not increase miRNA expression in CD4+ T cell clones in the absence of drug or in the presence of cyclosporine-A.

In the next component of the study, we evaluated miRNA expression in activated PBMCs from patients with different forms of cutaneous hypersensitivity associated with the different antibiotics; sulfamethoxazole and piperacillin and the anticonvulsants lamotrigine and carbamazepine. MiR-18a, miR-21, and miR-155 were studied, because they showed the higher levels of expression in the experiments among different TCC. Given the low frequencies of drug specific T cells in peripheral blood and the heterogeneity of their patient cohort, we decided to analyse the expression of miRNAs at two time points 24 and 48 h. PBMCs from patients were stimulated to proliferate *in vitro* in the presence of the culprit drug. Similarly, up-regulation of all three miRNA was detected. MiR-21 expression was up-regulated to the greatest extent in most patients at 24 h, while miR-18a showed higher expression after 48 h. MiR-155 expression was elevated at both time points. The expression of this miRNA was drug - and time – dependent supporting again that this miRNA is expressed after drug challenge. Thus, it might be possible to use miR-155 as an early diagnostic biomarker of drug hypersensitivity reactions.

This work is important in several ways in the context of this thesis, and the investigation into the mechanistic evaluation of DHR in prediction and diagnosis. Two issues with the LTT as a diagnostic tool of DHR are the time taken for the assay to be completed (6 days), as well as the number of cells required to perform the assay. The time taken is important as the accurate diagnosis of the culprit drug in a DHR is extremely time-sensitive in order to know the correct course of action for treatment of

the patient, and in order to ensure that their regular treatment schedule is not interrupted as manifestations of their original disease can become harmful during extended period of treatment cessation. As explained by chapter 5, the number of cells needed for an assay (particularly for paediatrics) is often a limiting factor in both the amount and quality of data obtained. The assay developed for the work carried out in this chapter has taken both facts into account in that it takes only three days from the sample being taken, to the results being obtained (as opposed to six). The miRNA up-regulation assay uses the same number of cells as the current form of the LTT; whilst this is not an improvement on the current LTT, fewer cells are needed than in other diagnostic assays such as the ELISpot. An important future experiment would be to assess the exact number of cells necessary for extraction of the minimum amount of usable RNA for assessment of miRNA up-regulation in response to drug challenge. We can now use the knowledge obtained from this chapter (as well as all other thesis chapters on T cell priming, immune regulation, and the assessment of the LTT) to aid in the development and optimisation of the assays utilised for the experiments carried out in the process of this thesis. The use of miRNA and detection of immune regulation at the transcription level is an important development in the understanding of immune activation and the initiation of a DHR; and can play an important role in their diagnosis. However, further studies to understand the complexity of correlation between T cell proliferation and miRNA up-regulation and how this may affect DHR patient diagnosis must be performed before any miRNA up-regulation assay can be used widespread. It is also important to note that we must be able to assess the quality of the assay in as many different classes of drugs as possible, as current in vitro tests' major pitfall is their lack of versatility amongst different drugs used and patient populations. Another interesting experiment that could be performed to understand the

regulatory effects miRNAs elicit on immune activation would be to isolate miRNAs as we have done in this chapter, and then add them into the T cell priming assay such as the T-MWA to see their effects on T cell priming. This could then be used in conjunction with flow cytometry to see the effects of the miRNAs on other immune regulation mechanisms such as Treg function, and the immune checkpoints PD-1 and CTLA-4. One potential drawback of the miRNA up-regulation assay is the difference in optimal incubation time of cells with drug for the up-regulation of different miRNAs. This means that we would need to perform further experiments to understand whether 24 or 48 h are better incubation times for each individual drug to be tested, and for each miRNA to be studied.

To conclude, our results clearly show a change in the levels of expression of miRNAs in activated CD4+ T cells from hypersensitive patients after drug challenge. These results contribute to the current understanding of the role of miRNAs in cutaneous reactions by demonstrating that they could originate from drug-specific T cells during activation. However, how these miRNAs expressed in drug-specific T cells end up in the plasma or skin altering cellular and tissue physiology in drug hypersensitive patients' needs to be further investigated. Importantly, this research opens a new field to investigate the use of miRNAs as biomarkers for *in vitro* diagnosis of drug hypersensitivity reactions. Whilst the use of the *in vitro* miRNA up-regulation assays for the assessment of DHR has been shown in this chapter to be a promising tool; further investigations must be done before we can say it is a marked improvement over the currently established methods such as the LTT and ELISpot.

7 FINAL DISCUSSION

Firstly, it is important to understand the aims of the experimental chapters, and evaluate whether or not they were accomplished in this thesis. The main aims of chapter 3 were to develop a predictive screening system that can detect the number of donors responding to a drug, as well as the number of cells in each donor. This was mostly achieved in that the T-MWA is able to determine the number of responsive cells in multiple donors on the same plate. However, the T-MDA still needs some work to be able to accurately predict the number of responsive donors out of a panel. Chapter 4 had the aims of determining T reg abundance, PD-1 and CTLA-4 expression and their functional effects on naïve T cell priming in response to different drug treatments. These aims were all achieved expect that we were not able to successfully determine the functional effects of Treg generation upon different drug treatment. In chapter 5, the LTT was to be evaluated as a tool for the prediction of DHR in paediatric patients to determine if it was fir for purpose. This aim was achieved; the LTT was found as not fit for purpose. I was able to use this data in order to formulate new ideas to improve the current LTT assay. Finally, the main aims of chapter 6 were to correlate the upregulation of T cell activation linked miRNA and T cell proliferation to determine if this is a viable alternative to the LTT. Again, the aims of this chapter were met as four different miRNAs (especially miR-155) were found to correlate with T cell proliferation in response to drug treatment. This new assay could be a viable alternative to the LTT as it can show similar results, but in a 3-day shorter timescale.

In recent years adverse drug reactions are having an increasing burden on the healthcare system; with close to 7% of hospital admissions (over 100,000) being due to adverse drug reactions and drug hypersensitivity reactions [1,2]. Indeed, many of these reactions can be attributed to the sheer number of novel treatment strategies being

developed to a multitude of illnesses [1]. Type A ADRs can be more easily prevented as they are on-target adverse events usually influenced by dosage and regimen. However, type B reactions are an increasing problem for both the healthcare systems, and for drug development as they are off-site adverse events that are not predictable from the pharmacology of the treatment drug; type B has also been referred to as 'bizarre' [5]. This makes them difficult to predict and prevent. These types of reactions can include severe reactions with high morbidity and mortality rates such as SJS/TEN [156,274,333]. In addition to the exorbitant cost burden to the healthcare systems, ADRs have a large effect on the drug development process, as ADRs are one of the major contributors to the well-known high rate of late-stage attrition in the R&D process after millions of pounds have already been spent [334,335]. This often occurs frustratingly late in the development process, where withdrawn drugs would otherwise have been useful (and in some instances life-saving) treatment strategies for a large cohort of patients. As these reactions are unpredictable in nature and only occur in some individuals and not others, drugs often pass through the clinical trial stage, only to be found to cause ADRs in individuals post-release [336].

The main focus of this thesis was to further the understanding of immune mediated adverse events; drug hypersensitivity reactions (DHRs). As well as to develop and improve current predictive and diagnostic research tools for the study of DHR. There have been numerous investigations into the pathomechanisms of DHR since Gell and Coombes first attempted this in 1963 [337]. It wasn't until a few decades later that the now infamous categorisation of DHR into type I-IV was set out. Whilst many studies have focused on immediate reactions involving IgE and innate cell activation (such as mast cells); the pathomechanisms of delayed type IV DHR have yet to be fully elucidated. The Gell and Coombes classification has been re-interpreted by numerous

scientists over the years due to the discovery of new types of DHR, and new subsets of detectable antibodies and antigen-specific T cells that that do not fit within the confines of the system set out by Gell and Coombes' initial interpretation. This includes the expansion of type IV DHR by Pichler in 2003 to include subsets a-d [338,339]. Such is the complexity and fast-moving nature of the research into immunology and DHR specifically, even these expanded classifications are now outdated by the discoveries of novel immune cells implicated in the pathomechanisms of DHR such as T helper subsets T_h9/17/22 [50].

Other investigative work into the reasons for DHR have led to the discovery of HLA risk allele associations. This implicates proteins expressed on the surface of the HLA-allele that interact specifically with a drug of interest to activate T cells. Whilst GWAS studies have uncovered many associations between certain risk alleles and a number of drugs [340], further investigations have discovered that most of these associations are loose at best, and indeed the majority of people who possess the risk allele do not actually present with DHR. Due to poor PPV and NPV of HLA-risk alleles as predictors of DHRs, most associations have not been clinically implemented as pre-screening prior to drug treatment. Abacavir's association with HLA*B-57:01 has been robust enough to be implemented clinically. Further experiments into this association have further defined this association as an important one [125–127], and even led to the discovery of a new mechanism of T cell activation (altered peptide repertoire). Whilst HLA risk allele associations may play a role in the pathomechanisms of DHR; it is clear that it is not the only defining feature, and certainly cannot be used in isolation to predict DHR. For this reason, studies in this thesis aims to better interpret the likelihood of immune activation by a compound/drug at the cellular level.

The *in vitro* assessment of the likelihood of DHR, as well as diagnosis of DHR, has been attempted in numerous formats. The CD69 upregulation flow cytometry assay aimed to detect the activation of basophils and T cells by their cell surface marker for activation. This is less invasive than traditional *in vivo* tests such as intradermal testing, and can provide increased sensitivity over tests against immediate type DHR through IgE detection. However, the utility of this test can vary wildly dependent on the class of drug being investigated [238,239].

The naïve T cell priming assay developed by Faulkner *et al.,* in 2012 is currently the method used most frequently within our lab to attempt to understand the likelihood that a drug or compound will induce T cell activation [71]. Sulfamethoxazole-nitroso (our model hapten – induces responses in 9/10 individuals), Bandrowski's base, piperacillin, flucloxacillin, dapsone/nitroso-dapsone and more recently ticlopidine have been used successfully in the T cell priming assay, with antigen-specific positive proliferative responses detected [28,135,137,144,225,232,296,341]. As recently as 2019, nickel (a contact allergen) has been investigated using a naïve T cell priming assay, with both CD4+ and CD8+ T cell responses involved in the observed antigen specific proliferative responses [342].

Despite the many successes of the naïve T cell priming assay, there are some limitations that needed to be addressed. There are many different steps involved in the priming assay; from PBMC isolation, cell subset magnetic separation, CD14+ monocyte to moDC culture, co-culture of moDCs, naïve T cells and antigen, and readout proliferation assay. Each of these up to now were performed in separate plates, with cells being moved from one plate to another between each individual step, making the priming assay more labour intensive, susceptible to mistake and cellular damage or contamination. Another problem that could be stated for the original

priming assay set out by Faulkner *et al.,* was that thus far, the drugs that provided the best responses in the assay were HLA class I restricted; with HLA class II restricted drugs often resulting in negative priming. One reason for this could be a low precursor frequency of circulating antigen-specific naïve T cells to certain drugs [256,257]. In a priming assay with readouts only performed in triplicate, the precursor frequency of primed T cells was not reached, leading to negative priming. Another limitation was that due to the numbers of cells needed for a single assay, only one donor per plate could be assayed at any one time. The assays developed in this thesis – the T-MWA and T-MDA – have served to mitigate these issues. Both the T-MWA and T-MDA are performed in a single 96-well plate after moDC culture and remain as such throughout the experiment, making them medium-throughput, and easier to manage than the previous T cell priming assay. The T-MWA was developed to take into consideration the naïve T cell precursor frequency, as 16 wells of negative control and 40 wells of drug treatment per donor are assayed. Finally, the T-MDA was developed to enable the screening of multiple donors in a single assay. This was achieved as cells were plated up in two lots of triplicate, with only the optimal concentration of drug being used for the drug treated wells. The above can be seen in section 2.5.5.

The use of the T-MDA with multiple donors was able to outline potential immune activation in the different donors on a single plate. The idea being that any donors flagging up a potential reaction could then be investigated further using the T-MWA. Although we weren't able to perform as many of these assays as we would have liked, the premise of the assay (cell ratios) remains similar to the T-MWA and thus any improvements that can be made for the T-MWA can also be considered applicable for the T-MDA. In the T-MWA we were able to successfully prime to SMX-NO, parent SMX, piperacillin and Bandrowski's base to different levels. We were also able to

show that the assay is repeatable, with similar levels of weak, good, and strong responses observed in the same donor with blood donations taken >6 months apart. The traffic light system enabled us to determine the different levels of response (weak, good, strong) and so determine the likelihood that an immune event may occur, as well as the severity of the response. The scatter plots allowed for statistical evaluation of the antigen-specific response, as well as look at the overall distribution of responses, well to well. As expected, the greatest proliferative responses were observed against SMX-NO and BB. Whilst reduced responses were observed against the other test compounds; this allows us to use SMX-NO and BB as positive controls as model antigens, measuring other antigen specific responses against them to investigate the potential for a novel compound to prime and activate naïve T cells. Whilst these results are promising, advances in the field explained in recent literature have provided some evidence that our assay can be improved upon. The characterisation of antigen-drug-peptides eluted from the MHC in instances such as HLA*B-57:01 [126] have provided evidence for the mechanism of activation of T cells. Unpublished data within our lab show that the characterisation and elution of other drug-modified peptides is possible. We hypothesise that priming with these peptides may help to elucidate immune activation in our T-MWA assays against difficult to prime to drugs. Traditionally, SMX-NO has been easier to prime to than the parent drug SMX. SMX-NO is formed from the metabolism of SMX to a hydroxylamine intermediate before nitroso metabolite is formed [111]. For this reason, it would be interested to create an assay in which we can culture naïve T cells, parent drug, and APCs along with hepatocytes. If cultured in the same well, T cell activation as well as drug toxicity will cause hepatocyte death. However, the use of a membrane plate system such as the IdMOC plate system (Sigma-Aldrich product code: Z687936) enables the culture of all of the

above, without the need for them to be in physical contact. The membrane allows for the free passage of drug and CRM post-metabolism. It would be interesting to use such a system to evaluate priming to parent drugs in the T-MWA that have metabolism as a prerequisite for immune activation. This could also be important as metabolic differences are observed between naïve and memory CD8+ T cells [343]. Another aspect of the priming of naïve T cells in our systems, are the ability of the moDCs to present the antigen to the naïve T cells in co-culture. In our assay, DCs are matured before co-culture is established. This does not take into account that the DC ability of antigen engulfment is better in immature DCs whereas antigen presentation ability is improved upon maturation [344]. Additionally, monocyte derived DCs have increased lysosomal degradation of antigen compared to human derived or mouse derived DCs [345]. For this reason, we could try to pulse the immature DCs with drug, and then mature the DCs after pulsing, to better take into account the different capabilities of DCs in different states of maturation. Recent studies have also uncovered that LKB1 is critical for DC activation of T cells through AMPK activation. Loss of LBK1 function can also enable Treg dampening of responses [346]. The role of LBK1 expression should be investigated in our T-MWA assays. Another potential point of interest in our priming assay is the use of 'naïve T cells', which are actually CD45ra+ lymphocytes. A new subset of T cells described as exhausted senescent T cells could be in our 'naïve' population, and thus be causing reduced response, or failed assays as they are functionally inactive. These cells are known as TEMRA (T effector memory reactivated cells) and are phenotypically different from 'true naïve' cells due to their lack of CCR7 [347]. It could be important to investigate the difference in results in the T-MWA when true naïve cells (CD45ra+CCR7+) are primed vs our traditional naïve population (CD45ra+) and the TEMRA subset (CD45ra+CCR7-).

Whilst the T-MWA was successful in terms of increasing the throughput and ease of use of the traditional priming assay, it was always clear that we must investigate whether or not we are replicating the correct immune microenvironment for priming to occur. Immune regulation such as the immune checkpoints, as well as the presence and function of Tregs needed to be investigated, especially due to their increasing involvement in cancer therapies (that sometimes lead to severe adverse events). Our experiments in chapter 4 showed that Tregs were being generated from the naïve population in 3 out of 3 tested donors during priming. Due to time constraints we were not able to complete mechanistic evaluation of the function of these Tregs within our priming cultures. If further time were available it would have been useful to remove the Tregs post-priming (before readout stage) to understand their immune-suppressive role in our priming assays. The role of co-inhibitory checkpoints are an important part of immune activation, with disrupted PD-1 implicated in autoimmunity [184], and the CD28 family interaction (including CTLA-4) being considered a prerequisite for immune response [348]. One explanation for why we observed different levels of priming dependent on the drug used was that the drug used may directly influence the expression of co-inhibitory receptors differentially. Therefore, we investigated the expression levels of PD-1 and CTLA-4 over the course of the priming co-culture with either SMX-NO or piperacillin. We were able to establish that under certain circumstances PD-1 expression and CTLA-4 expression were fluctuating at different rates dependent on the test drug used. Additionally, experiments in the chapter showed that blocking either of the co-inhibitory checkpoints could increase the intensity of proliferation observed (as well as background proliferation of T cells). It would be useful to continue research into the co-inhibitory checkpoints when priming against a larger range of drugs to uncover any patterns in expression and the direct effects this

has on our priming outcomes in the T-MWA. It could also be interesting to investigate the role of a wider variety of co-signalling molecules such as TIM-3, LAG3, and co-stimulation such as CD28. This knowledge could be applied not only to our T-MWA priming assays, but to diagnostic assays such as the LTT which was used in chapter 5.

Not only is the *in vitro* prediction of DHR an issue, but the diagnosis of such events can also prove problematic, especially for vulnerable patient populations such as children exposed to multiple drug regimen. Often, it is difficult to correctly pin-point the culprit drug causing DHR when more than one drug is prescribed concurrently. Indeed, in our own investigations in this thesis, there were cases where multiple potential drugs were flagged as positively activating T cells in paediatric patient samples, whilst in others, no drugs were considered to significantly activate T cells. Despite this, the LTT remains the gold standard for *in vitro* DHR diagnosis and is a marked improvement on some of the *in vivo* alternatives. Recent studies have shown that oral provocation tests resulted in more accurate outcomes than skin tests in children. Despite this, some adverse events occurred as a result of the testing [349]. Other studies have shown intradermal tests to perform well in the diagnosis of immediate adverse events to ceftriaxone, they performed poorly at detecting delayed responses [350], again outlining the importance for a safe, robust *in vitro* diagnostic test.

Some issues with the LTT include the time taken for the assay to be completed (6 days), as well as the blood volume needed to perform the assay being high for paediatric patients in particular. Most studies in this thesis were performed to investigate DHR at the cellular level, but relatively little is known about immune activation at the transcriptional level. miRNAs have become increasingly implicated as regulators of a range of biological processes; namely, the innate and adaptive immune system, and are known to be involved in auto-immunity [211]. For this reason,

an assay was devised whereby an LTT -like assay was set up on day 1, and after only 48 hours, cells were lysed and RNA was extracted before miRNAs known to be involved in immune regulation were detected by real-time qPCR. We were able to detect differential expression of miR-9, 18, 21, and 155 in T cell clones and hypersensitive patient PBMC, all of which had provided positive antigen-specific proliferation or ELISpot data. miR 155 was notable in our work, and has also been implicated in chronic inflammation in other studies that describe a miR-155 induced exhausted resident CD8 T cell population [351]. This provided credence to the theory that miR upregulation could be used to rapidly diagnose DHR *in vitro*. Further studies in the literature have shown miR expression to be involved in the regulation of T helper cells and Tregs, influencing immune activation. miR investigated in chapter 7 were detected in this study, in addition to other miR such as miR 214, 386 and others which should be investigated by further study within the lab [352]. Another potential target of interest is miR 210 which has been implicated in psoriasis-like inflammation through the induction of T_h1 and T_h17 T cell subsets [353]. Work should continue to fully-investigate the utility of the miR upregulation assay as an alternative to the gold standard LTT, especially for paediatric cohorts where rapid diagnosis is of extreme importance. This work, along with continuous developments implicating miR induced gene regulation on the function of macrophages, dendritic cells, T cells and B cells [354] mean we can also investigate the role of miR as immunological regulators in our T-MWA priming assays. A useful experiment would be to amplify the miR of interest, and then pulse APCs with the amplified miR, as well as adding them directly into the priming culture to investigate their immunoregulatory roles *in vitro*.

Throughout this thesis, we were able to improve *in vitro* assays for the prediction and diagnosis of DHR. The two newly developed assays; adapted from the original naïve

T cell priming assay, fulfilled their role, as we are now able to prime naïve T cells from multiple donors in a single plate in the T-MDA. Following on from that, we can then look more deeply into the antigen-specific mechanistic activation of T cells. The T-MWA enables us to look at the intensity of proliferation, as well as providing a scaling level of immunogenicity, all the while taking into account the naïve T cell precursor frequency for a drug. However, it must be stated that immune regulation plays a vital role in the activation of T cells, and thus, our studies in chapter 5 elucidated the generation of Tregs, as well modulation of co-inhibitory checkpoints PD-1 and CTLA-4 as potentially important factors in whether or not immune activation occurs.

We were also able to perform LTT as a diagnostic tool on paediatric DHR patient samples, and uncovered some of its limitations, namely, the time taken to perform the assay in such a time-sensitive environment. For this reason, we were able to devise the miR upregulation assay and establish connections between the upregulation of DHR-linked miR and T cell activation. This assay enabled us to decrease the time taken to perform the diagnostic assay from 6 days to 3. Additionally, this work uncovered another layer of immune regulation that is important in trying to understand naïve T cell priming at the transcriptional level. This work ties in nicely to one of the overarching important themes of this thesis – in trying to predict the likelihood that a drug or compound can activate T naïve T cells leading to DHR, are we replicating the correct immune microenvironment including immune regulation?

Whilst we were able to successfully bring about improvements to the *in vitro* assays available to us for the diagnosis and prediction of DHR, we believe that more detailed studies into immune regulation (studies carried out in this thesis and future ideas

generated above) could hold the key to our ability to accurately and consistently predict and diagnose these reactions *in vitro* – something that is desperately needed.

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